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(54) Title: A REVERSIBLE STOICHIOMETRIC PROCESS FOR CONJUGATING BIOMOLECULES

(57) Abstract

Compositions comprised of at least two biopolymers (e.g., nucleic acids or polypeptides), which are conjugated to an insoluble support by two different reversible linkages, which are cleavable under selective conditions, as well as methods and components for producing the same are described.

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A Reversible Stoichiometric Process for Conjugating Biomolecules

Background of the Invention

Methods for reversibly linking biomolecules (e.g. nucleic acids with reporter groups or to solid supports) is important for many applications in the life sciences; it is used amongst other applications in DNA sequencing, DNA diagnostics, nucleic acid purification, Polymerase and Ligase Chain Reactions (PCR, LCR), hybridization experiments and solid phase biochemistry. Most frequently, a reversible linkage is accomplished via a streptavidin-biotin interaction (L.G. Mitchel and C.R. Merril (1989) *Anal. Biochem.*, 178, 239-242; B.H. Bowman and S.R. Palumbi (1993) in E.A. Zimmer, R.L. Cann and A.C. Wilson (ed.) *Methods of Enzymology*, Academic Press, New York, Vol. 224, pp. 399-405; X. Tong and L.M. Smith (1992) *Anal. Chem.* 64, 2672-2677).

Another reversible linkage, which is particularly amenable for linkage of nucleic acids, can be accomplished via heterobifunctional trityl groups, which can be cleaved under acidic conditions (E. Leikauf, F. Barnekow and H. Köster, Heterobifunctional Trityl Derivatives as Linking Agents for the Recovery of Nucleic Acids after Labeling and Immobilization (1995) *Tetrahedron* 51, 3793-3802; H. Köster, J.M. Coull and B. Gildea, Succinimidyl Trityl Compounds and a Process for Preparing Same, Protecting Groups for Natural Products, US Patent 5,410,068).

The interaction of metal chelates with polypeptide sequences such as oligohistidine has been used for affinity chromatography of proteins (J. Porath (1992) *Protein Express Purif.* 3, 263-281; M.C. Smith et al. (1988) *J. Biol. Chem.*, 263, 7211-7215; E. Hochuli and S. Piesecki (1992) *Methods*, 4, 68-72; E. Huchuli et al. (1988) *BioTechnology 6*, 1321-1325; E. Blum et al. (1994) *Biochem. Biophys. J.* 29, 113-121; see also European Patent No. 0 253 303 to Hoffman LaRoche AG), nucleic acids (Ch. Min and G. L. Verdine, Immobilized Metal Affinity Chromatography of DNA (1996) *Nucleic Acids Res.* 24, 3806-3810) and recently a system to detect proteins has been

introduced (Qiagen (1996): QIAexpress Detection System). Occasionally also disulfide bridges are used, which can be cleaved under reducing conditions.

However, in applications in which proteins (e.g. antibodies, enzymes) are to be linked to nucleic acids (i.e. for the detection of nucleic acids), no specific and reproducible linkage to the nucleic acids can be established, due to the fact that during chemical functionalization or activation of functional groups on the surface of the protein, no precise selection of amino acid side chains is possible and therefore neither the attachment site nor the stoichiometry can be controlled. Therefore, the results obtained can be different from batch to batch which negatively influences the generation of quantitative nucleic acid detection systems. In addition, there is no control over whether the amino acid side chain is incorporated into the active site. These factors all reduce the technical value of such procedures.

The application of solid phase techniques simplifies the preparation and purification of the reaction products, which is important for subsequent analytical and biochemical procedures. Since in some cases cleavage of one of the products from the support is needed. e.g. for further biochemical reactions in solution or signal detection, a combination of at least two different reversible linkages cleavable under mild and selective conditions is needed.

Summary of the Invention

In one aspect, the invention features compositions comprised of at least two biopolymers (e.g. nucleic acids or polypeptides), which are conjugated to an insoluble support by two different reversible linkages, which are cleavable under selective conditions.

In another aspect, the invention features novel methods and components for specifically conjugating biomolecules under completely controlled stoichiometry based on the specific and strong interaction between chelators in the presence of metal ions. In one embodiment, imidazolyl moieties are introduced via the introduction of

histidine residues (e.g. oligo-His) into a polypeptide (e.g. by recombinant DNA techniques). The oligo-His polypeptide can then interact in the presence of a metal with a nucleic acid carrying a chelator functionality at a position which is exposed and does not interfere with Watson-Crick base pairing of the nucleic acid. In another embodiment, which is particularly well-suited for the attachment of biomolecules other than polypeptides or for the reversible immobilization of nucleic acid molecules, the nucleic acid can carry a series of imidazolyl functionalities in a format which makes them available for chelation and which does not interfere with Watson-Crick base pairing; in which case, the other conjugating partner molecule can carry the chelator functionality.

By combining this reversible concept with other reversible or irreversible linkages, novel biochemical formats including diagnostic assays are possible in which favorable solid phase procedures are coupled with sensitive detection principles.

Brief Description of the Figures

Figure 1 (a) and (c) pictorially depict two general approaches of the invention in which a spacer molecule, A, linked to a polymer support, P, forms a reversible linkage, I, to a nucleic acid or protein/peptide molecule, B, which itself is linked by another reversible linkage, II, to either a nucleic acid, protein/peptide or small molecule (e.g. reporter molecule). Linkage I can be a heterobifunctional trityl group or a hydrophobic interaction stable under aqueous conditions or a photocleavable bond and II can be a bond, which is generated through a chelate complex. The two parts which form the linkage can be reversed (I', II') as shown in (b) and (d).

Figure 2 schematically depicts a nucleic acid molecule, B, which is linked through a spacer, A, via a reversible linkage, I, to a polymer support, P. B interacts via Watson-Crick complementarity with a nucleic acid molecule, C, which in turn through another reversible linkage II allows interaction with a reporter functionality D which can be a protein (enzyme), a nucleic acid or a small detector molecule.

Figure 3 schematically depicts the same approach as in Figure 2 with the

exception that B is linked to the polymer support through a spacer A with a non-reversible linkage.

Figure 4: shows an example of the chelate complex formed between a six residue histidine (his_6) tail and nitrilotriacetic acid (NTA) in the presence of Ni^{2+}.

Figure 5 schematically depicts a reaction, wherein a synthesized, protected N,N-dicarboxymethyl-serine phosphoamidite is synthesized as a chemical building block to introduce the NTA functionality into synthetic oligonucleotides.

Figure 6 shows the synthesis of a chelate-linked oligonucleotide to a his_6-BAP (bacterially generated alkaline phosphatase) conjugate by use of the phosphoamidite chelate precursor.

Figure 7 shows the synthesis of a chelate-linked oligonucleotide to his_6-BAP conjugate via retritylation and subsequent substitution with a chelate building block.

Figure 8 shows the structure of imidazolyl phosphoamidite building blocks for the single or multiple addition of an imidazolyl moiety during chemical oligonucleotide synthesis.

Figure 9 depicts the introduction of an imidazolyl moiety through an imidazolylnucleoside phosphoamidite.

Figure 10 shows the introduction of multiple imidazolyl moieties through chemical peptide synthesis of oligohistidine onto an oligonucleotide during solid phase chemical synthesis of oligonucleotides.

Figure 11 shows the chelate modified uracil and adenine nucleoside triphosphates for the enzymatic introduction of chelate functionalities into nucleic acids. Corresponding derivatives can be envisioned for cytidine, guanine or modified

nucleosides.

Figure 12 shows imidazolyl modified uracil and adenine nucleoside triphosphates for the enzymatic introduction of imidazolyl moieties into nucleic acids. Corresponding derivatives can be envisioned for cytidine, guanine and modified nucleosides.

Figure 13 schematically depicts solid phase separation/detection using NHS-DMT oligonucleotides linked to a solid phase and subsequently linked to a BAP-his_6 detector molecule via the LCR (Ligase Chain Reaction).

Figure 14 schematically depicts the detection of Polymerase Chain Reaction (PCR) products via the process of the invention.

Detailed Description of the Invention

As shown in Figure 1, two different reversible linkages I and II (a,c), which could be positioned with their functionalities reversed (I',II'; b, d) are used to link "biomolecules" or "biopolymers" (i.e.organic molecules, including nucleic acids, peptides, polypeptides). to an insoluble support. The circled P represents an insoluble or solid support.

"Insoluble supports" or "soluble supports" as used herein can be flat such as membranes, glass plates, metals, plastic films and composites thereof with a homogeneously functionalized surface or functionalized to result in an array format including flat supports with pits, wells, combs, microtiter plates, microtiter filter plates; flat supports can also be magnetic or with an array shaped (checkered) magnetic field; solid supports can also be used as beads from different plastic materials, inorganic supports such as silica, GPG (Controlled Pore Glass), metal, different polymeric material, cellulose, Sephadex, Sepharose; the beads can be porous or non-porous, of different diameter and magnetic or non-magnetic. Also a combination of beads in the pits/wells of flat supports thus forming an array format can be employed.

Compound A can be a spacer, a nucleic acid sequence (or nucleic acid analog/mimetic) or a protein or peptide sequence, B can be a nucleic acid (or a nucleic acid analog/mimetic) or a peptide or protein, whereas C can be nucleic acid (or a nucleic acid analog/mimetic), protein/peptide or a small reporter molecule. As an example A is a spacer and I is a heterobifunctional trityl group which is coupled to a nucleic acid B; B carries a chelate functionality which interacts with the poly-his tail of a recombinant alkaline phosphatase (his_6-AP), which carries e.g. a sequence of six histidine residues at the C-terminal end of the polypeptide chain. If a chromogenic or fluorogenic substrate is added, for example, dephosphorylation generates color or light thereby providing a nucleic acid detection system. The advantage of this system is that the detection can be done either on the insoluble support or after releasing B from the support by cleavage of bond I (or I'). It is therefore possible to remove all side-products from a reaction by filtration due to the attachment to a solid phase before performing the analytical step in solution. This leads to a robust, reproducible performance.

Figure 2 shows schematically how amplification (e.g. polymerase chain reaction (PCR) or ligase chain reaction (LCR) products B-C can be captured specifically, purified and subsequently detected on the support or in solution. The first reversible linkage I (or I') e.g. a heterobifunctional trityl group anchors one strand of the LCR or PCR product via a spacer A to the support through an acid labile tritylether bond the precursor of which has been introduced by an appropriately functionalized primer during the LCR or PCR reaction. The strand C carries e.g. the chelate functionality also introduced by using an appropriately functionalized primer during PCR or LCR. The chelated moiety can then interact with a reporter functionality e.g. his_6-AP for subsequent detection and quantification of amplification product. B can also be a cDNA molecule which can be linked through its 5'-end to the polymer support. With appropriate primers, solid phase DNA sequencing can be performed. Considering an array format, this could be used for high throughput genetic and expression profiling experiments.

As shown in Figure 2, B could also be a specific (or oligo-dT) capture

sequence to fish mRNA. The cDNA can be directly synthesized since the capture sequence simultaneously can act as a primer for the RNA dependent DNA polymerase. The RNA can be removed, the cDNA purified by washing and filtration steps and either released or directly used for subsequent DNA sequencing. It can also be envisioned that the capture sequence while serving as a primer for the RNA dependent DNA polymerase can be used directly to generate sequencing ladders employing ddNTP's as terminators. After purification of the sequencing ladders by washing and filtration, the bond to the polymer support is cleaved and the purified sequencing ladders subjected to either gel electrophoretic or mass spectrometric separation (H. Köster et al., A Strategy for Rapid and Efficient DNA Sequencing by Mass Spectrometry, *Nature Biotech*, (1996) 14, 1123-1128; U.S. Patent No. 5, 547,035 to H. Köster; International Patent Application No. W094/21822 to H. Köster; and International Patent Application No. W096/29431 to H. Köster)

Figure 3 shows a simplified version of Figure 2 in that nucleic acid fragment B is immobilized through a non-reversible bond via a spacer A to the solid support whereas nucleic acid C carries the reporter functionality via a reversible linkage so that detection can be performed either on the support or in solution.

In Figures 1-3, biopolymer C or D could be synthetic peptides linked to an immobilized nucleic acid B or B-C respectively via a reversible linkage as described (heterobifunctional trityl, photocleavable, chelate, hydrophobic interaction) which is then detected by mass spectrometry. Various defined peptide sequences can form a specific mass tag which can be used as a specific nucleic acid identifier. Conversely specific nucleic acid sequences can be used as mass tags (specific identifiers) for proteins immobilized through a spacer A.

For use in the instant process, nucleic acids can be single stranded or double stranded polynucleotides (including oligonucleotides), whether natural or synthetic, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) or DNA/RNA hybrids, DNA containing ribonucleotides and/or dideoxyribonucleotides and

RNA containing deoxyribonucleotides. Also encompassed by the term "nucleic acid" are modified nucleotides (e.g. phosphorothioate modified) as well as nucleic acid mimetics or analogs, such as peptide nucleic acids (PNAs).

As used herein, the terms "protein", "polypeptide" or "peptide" are all used interchangeably to refer to gene products. Proteins can be antibodies, enzymes, receptor molecules; peptides could be of natural or synthetic origin with oligo-his tail, a functionality for hydrophobic interaction, a photocleavable functionality or chelator functionality and displaying different properties such as being adhesive or representing specific ligand-receptor or specific protease cleavage sites.

As used herein, the term oligo his tail or poly his tail refers to a chain of conjugated histidine residues. Preferred oligo his tails contain 2-10 histidine residues. Particularly preferred oligo his tails are in the range of about 4 to about 8 his residues. Reversible linkages can be formed by hydrophobic interaction between e.g. a trityl group (i.e. with long aliphatic alkyl chains) and a long aliphatic chain e.g. attached to a polymer support or a hydrophobic polymer surface such as that of polystyrene. Since most biochemical and molecular biological reactions are performed in aqueous solution such hydrophobic interaction might be of sufficient stability. Addition of organic solvents such as alcohols, acetonitrile, N.N-dimethylformamide and the like will destabilize (if necessary in conjunction with heat) the hydrophobic interaction and release the attached molecules.

A reversible linkage which can independently be addressed could also be a functionality which is cleavable under photolytic conditions (see e.g. J. Olejnik, E. Krzymanska-Olejnik and K.J. Rothschil, Photocleavable Biotin Phosphoramidites for 5'-End-labeling, Affinity purification and Phosphorylation of Synthetic Oligonucleotides (1996) *Nucleic Acids Res.*, 24, 361-366). If the wavelength needed for photocleavage is in the range of the laser wavelength used in MALDI mass spectrometry, this bond can be cleaved during mass spectrometric signal acquisition.

A reversible linkage can also be formed from a chelator functionality which interacts with another chelator (e.g oligo-imidazolyl or other oligopeptide moieties) in the presence of a metal ion. The term "chelator" refers to a single molecule, which comprises at least two Lewis basic atoms that are capable of associating simultaneously with a Lewis acidic atom, moelcule or ion-- either simple or complex. "Lewis base" is an art recognized term that refers to chemical moieties, which are capable of donating to another atom or moiety at least one pair of unshared electrons. Examples include uncharged functional groups such as alcohols, ethers, carbonyls, thiols, sulfides, amines, imines, and pyridine and imidazole nitrogens; and charged functional groups, such as alkoxides, thiolates, carboxylates and a variety of other anions. "Lewis acid" is an art recognized term that refers to chemical moieties, which are capable of accepting from another atom or moiety (e.g. a Lewis basic atom or moiety) at least one pair of unshared electrons. Examples of Lewis acid moieties include transition metal halides, with at least one vacant d orbital, alkali metal cations, alkaline-earth metal cations, and trivalent boron or aluminum compounds. A "bidentate chelator", "tridentate chelator" and "tetradentate chelator" refers to chelators comprising two, three and four Lewis basic moieties, respectively, capable of simultaneous donation of at least an equal number of unshared electron pairs to another atom, ion or moiety.

Figure 4 depicts a specific example in which the chelator functionality is a nitrilotriacetic acid (NTA) which coordinates with divalent metal cations such as Ni^{2+} and forms a strong complex with six imidazolyl groups from a his_6 tail linked to one of the conjugating partner molecules. The term "imidazolyl residue" or "imidazoyl group" refers to any substituted or unsubstituted form of imidazole (i.e. 1,3-diaza-2,4-cyclopentadiene). For example, the side chain of the amino acid histidine comprises an imidazolyl residue.

The determination of which of the two necessary functions is attached to the nucleic acid molecule or the protein depends on the ease and convenience of introduction of either functionality (e.g. NTA or his_6 tail). In case of proteins the site-specific introduction of a chelator molecule seems to be difficult whereas the his_6 tail can

be introduced through recombinant DNA technologies. In contrast to currently available procedures, for linking nucleic acids to proteins (e.g. chemical linkage using either maleimide-thiol coupling (S.S. Gosh et al. (1990), *Bioconjugate Chem.* 1, 71-76), disulfide bonds (B.C.F. Chu L.E. Orgel (1988) *Nucleic Acids Res.* 16, 3671-3691) or mediated via streptavidin, which binds both biotinylated nucleic acids and biotynylated alkaline phosphatase (AP) (J.J. Leary et al. (1983) *Proc. Natl. Acad. Sci.* USA 80, 4045-4049)), the introduction of the his_6 tail through recombinant DNA technologies allows site-specific introduction.

As an example which does not limit the scope of this invention, the process is explained for alkaline phosphatase (AP) as protein. Alkaline phosphatase (EC 3.1.3.1) is a versatile enzyme for many molecular biological applications. It catalyzes the hydrolysis of ester bonds in phosphomonoesters and is used in recombinant DNA technology to remove 5-phosphate groups from DNA fragments to prevent self-litigation of vector DNA molecules. Coupled to antibodies or oligonucleotides, it replaces radioactively labeled compounds by serving as a reporter and signal amplifying enzyme which cleaves chromogenic or fluorogenic substrates in diagnostic applications for the specific detection of DNA (Southern blot: E.M. Southern (1975) *J. Mol Biol.* 98, 503-517) or proteins (Western blots: W.N. Burnett (1981) *Anal. Biochem.* 112, 195-203).

Predominantly, AP is isolated from calf intestine (CIP) or the bacterium E coli. (BAP). AP consists of a homodimer. The stability of the enzyme, of advantage in diagnostic applications, can lead to severe problems in cloning experiments. Residual AP activity from the dephosphorylation of vector DNA can result in dephosphorylation of the DNA to be inserted so that no or only low yields of ligation products are obtained. Heat inactivation very often is not sufficient so that time-consuming removal is necessary using treatment with proteinase K and subsequent extraction from phenol/chloroform. This lengthy procedure will also drastically reduce the yield of the product. Alteratively, AP isolated from species living at low temperatures (shrimps) are employed; here heat inactivation is possible, however, reduced stability is disadvantageous for diagnostic applications.

A modified BAP derived from E. coli was genetically designed with a his_6 tail at its carboxy terminus. The his_6 tail was introduced using inverse PCR by which six histidine codons followed by a stop codon were placed at the 3' end of the gene (E. Blum et al. (1994) *Biochem Biophys J.* 29, 113-121). To achieve high expression levels of the recombinant enzyme in E. coli, the region coding for the signal peptide of AP together with the untranslated 5' and 3' regions were exchanged with homologous sequences from the E. coli ompA gene. The expression of the resulting protein construct was under the control of the IPTG (β-D-isopropyl-thio-galactoside) inducible ptac-promoter.

The BAP-his_6 synthesized in the E.coli cell can easily be isolated from an unpurified cell extract through affinity chromatography using commercially available Ni-NTA resins (Qiagen) to which it forms a strong and specific chelate complex via its his_6 tail. The modified enzyme is therefore now available in high yields, high purity and reproducible batch-to-batch quality. As part of the inventive process, BAP-his_6 is able to form with chelate-modified nucleic acids, a stable complex which for the first time makes available specific conjugates between proteins (here BAP) and nucleic acids in a reproducible 1:1 stoichiometry.

When peptides are generated by chemical synthesis, the his_6 tail can be directly incorporated during peptide synthesis. Chemical synthesis of peptides also allows the alternative approach in which a chelator functionality is attached to the synthetic peptide either at the N- or C- terminus or one of the side chains depending on which part of the peptide sequence is needed for the biochemical function.

The nucleic acid molecule can be functionalized either with the imidazoyl moieties or with the chelator functionalities. In case of synthetic oligonucleotides the chelator functionality can be introduced in different ways. An amino acid such as serine, cysteine or lysine can be transformed into a β-cyanoethylphosphoamidite (N.D. Sinha, J. Biernat, J. McManus and H. Köster (1984) *Nucleic Acids Res.* 12, 4539-4477) carrying a precursor of the chelator functionality (e.g. NTA as described in Figure 5 and 6 with serine as starting material). During deprotection after solid phase oligonucleotide

synthesis, the three carboxyl groups are liberated forming a NTA (nitrilotriacetic acid) group linked through a phosphodiester bond to the oligonucleotide chain. In yet another way, Figure 7 shows the introduction through a heterobifunctional trityl group. The oligonucleotide is, after regular final detritylation, retritylated with a heterobifunctional trityl group bearing an active ester moiety derived from either e.g. N-hydroxysuccinimide or employing active esters such as p-nitrophenyl esters. The active ester functionality is then reacted with a chelator molecule derived from e.g. lysine.

The imidazolyl functionality can be introduced during oligonucleotide synthesis employing an appropriate β-cyanoethylphosphoamidite as shown in Figure 8; single or multiple imidazolyl residues can be incorporated. A imidazolylnucleoside as shown in Figure 9 or a histidine peptide sequence covalently attached to the oligonucleotide chain (Figure 10) can also be used to introduce the necessary imidazolyl moieties for interaction with the chelator functionality.

The chelator and oligoimidazolyl functionalities can also be introduced in high molecular weight nucleic acids using either DNA dependent DNA or RNA polymerases or RNA dependent DNA polymerases using appropriately modified nucleoside triphosphates (either NTPs, 2'-dNTP, 3'-dNTPs, ddNTPs) as depicted in Figure 11. The base will carry either the chelator or the oligoimidazoyl functionality (Figure 12) in case of pyrimidine bases at C5 and in case of purine bases at C8 so that Watson-Crick base pairing is possible. Using the appropriate nucleoside triphosphates those functionalities can either be introduced internally (NTP for RNA synthesis or 2'-dNTP for DNa synthesis) or at the 3'-end (3'-dNTP for RNA synthesis, ddNTP for DNA synthesis). The incorporation can be performed during amplification procedures such as PCR, SDA or during DNA sequencing. Those skilled in the art will realize other approaches to introduce either chelator or oligo-imidazolyl moieties into nucleic acids.

Detection of the immobilized nucleic acid-protein/peptide conjugates can be achieved either directly on the polymer support or after selective cleavage of either reversible bond I (I') or II (II'). The signal can be detected by any of a number of means

including radioactivity, fluorescence, chemiluminescence (using e.g. 1,2-dioxetan derivatives) or colorimetric (using e.g. BCIP/NBT) methods depending on the substrates used as C or D Fig. 1, 2 and 3). D can be an enzyme such as AP which triggers upon contact with a substrate through its enzymatic activity the signal generation. C and D can also be detected through their molecular weight by employing mass spectrometric methods. Preferred mass spectrometer formats for use in analyzing the translation products include ionization (I) techniques, including but not limited to matrix assisted laser desorption (MALDI), continuous or pulsed electrospray (ESI) and related methods (e.g. Ionspray or Thermospray), or massive cluster impact (MCI); these ion sources can be matched with detection formats including linear or non-linear reflectron time-of-flight (TOF), single or multiple quadrupole, single or multiple magnetic sector, Fourier Transform ion cyclotron resonance (FTICR), ion trap, and combinations thereof (e.g., ion-trap/time-of-flight). For ionization, numerous matrix/wavelength combinations (MALDI) or solvent combinations (ESI) can be employed. Subattomole leels of protein have been detected, for example, using ESI (Valaskovic, G.A. et al., (1996) Science 273: 1199-1202) or MALDI (Li, L. et al., (1996) J. Am. Chem. Soc. 118:1662-1663) mass spectrometry.

The process of the invention is further demonstrated by solid phase separation and detection of Ligase Chain Reaction (LCR) products as seen in Figure 13 and products of PCR reactions (Figure 14). To those skilled in the art it is obvious that all applications and variations of amplification procedures including those useful for the detection of mutations and DNA/RNA sequencing are all adaptable to the process of the invention thereby significantly improving such processes.

The present invention is further illustrated by the following Examples, which are intended merely to further illustrate and should not be construed as limiting. The entire contents of all cited references (including literature references, issued patents, published patent applications and co-pending patent applications, as cited throughout this application) are hereby expressly incorporated by reference.

Example 1 BAP-his_6 Fusion Protein

The phoA gene coding for the BAP of E. coli (P.E. Berg (1981) *J. Bacteriol.* 146, 660-667; C.N. Chang et al. (1986) *Gene* 44, 121-125) was derived from E. coli strain HB101. The his_6 fusion at the carboxyterminus was generated via inverse PCR with six his codons followed by a stop codon derived from plasmid pHis 1. (E. Blum et al. (1994) *Biochem. Biophys. J.* 29, 113-121).

To increase the expression rate of the recombinant BAP-his_6 protein, its reading frame was embedded in the untranslated regions of the E. coli ompA gene (Chen et al. (1991) *J. Bacteriol.* 173, 4578-4586), coding for protein OmpA, which is a major protein constituent of the outer membrane in Gram-negative bacteria. In addition, the signal peptide of BAP (H. Inouye and J. Beckwith (1977) *Proc. Natl. Acad. Sci. USA* 74, 1440-1444) and the first two amino acids of the mature protein were replaced by the OmpA leader peptide and the first amino acid residue of mature OmpA, resulting in a mature chimeric BAP with the amino acid alanine instead of arginine-threonine at its N-terminus.

To bring the expression of the chimeric BAP-his_6 under the control of IPTG inducible chimeric tac-promoter (T. Amann et al. (1983) *Gene* 25, 167-178), a 2.5 kb *EcoRI-PstI* fragment containing the complete open reading frame of the ompA-phoA chimera) and the untranslated regions from the ompA gene was cloned into the expression vector pHK236 (a derivative of pJF118u: Fürste et al. (1986), kindly provided by M. Kröger, Giessen) to generate the BAP-his_6 expression plasmid vector pBAPHIS8. Expression is achieved by induction of logarithmic E. coli culture harboring plasmid pBAPHIS8 with IPTG in a final concentration of 1 mM for 2 h under shaking in a 37°C incubator. Isolation of BAP-his_6 is carried out according to developed protocols on Ni-NTA-Agarose (E. Hochuli et al. (1987) *J. Chromatography* 411, 177-184).

Example 2 Dephosphorylation of DNA Fragments with Solid Phase Bound BAP-his_6

A solution containing DNA fragments is incubated with beads carrying immobilized metal ions complexed with BAP-his_6 protein. To remove the enzymatic activity after the reaction is carried out, filtration or centrifugation removes beads with adsorbed enzyme. Alternatively, a solution containing DNA fragment can be filtered through a derivatized membrane, carrying immobilized metal ions complexed with BAP-his_6 protein.

Example 3 Detection of LCR Products in Microtiter Filter Plates

The use of BAP-his_6 as a reporter enzyme for LCR is carried out in the wells (96 or more) of a microtiter filter plate (MTFP) with 96 samples with oligos A-D (Figure 13). One of the oligos (oligo A being the marker oligo, Fig. 13) carries at its 5'-end a chelating group. In the presence of a template DNA the marker oligo is incorporated into one strand, the marker strand, consisting of oligos A and B, with B ligated to the 3'-end of oligo A. Under denaturing conditions (or after denaturing), ligation products, oligos and other smaller by-products are transferred by suction into a second MTFP with a derivatized filter membrane. To this filter, oligo D or part of it with sequence complementary to oligo B is coupled via NHS-DMT (heterobifunctional trityl derivative) linkage. Hybridization occurs between membrane bound oligo D and oligo B or the marker strand AB. After removal of supernatant and washing, only oligo A incorporated in the marker strand AB by ligation remains in the wells of the MTFP. BAP-his_6 and a divalent cation such as Ni^{2+} are incubated in the wells under adequate conditions to allow coupling of BAP-his_6 to the marker strand. After removal of unbound BAP-his_6 by washing and filtration, chromogenic or fluorescent AP substrates are added. Only wells containing the LCR product show AP activity as a positive result, bound D alone or the single strand CD cannot give rise to any signal. The experimental setup allows multiplex LCR by employing a mixture of oligos in the LCR and subsequent transfer of the LCR products by suction through a stack of different MTFP with specific bound oligo sequences. This experiment setup is amenable to automation, since the

reaction can be carried out e.g. in filter tubes or filter plates, which allow removal of contaminating agents, buffer changes and even detection *in situ* by dispensing and filtration of different liquids.

Example 4 Sequence Specific Detection of PCR Fragments

PCR is carried out in crude cell lysates with a derivatized oligonucleotide primer (Figure 14). After denaturing, the PCR reaction is filtrated through a membrane derivatized with a capture oligo. It can contain any sequence, which is complementary to the expected PCR fragment and hybridizes with strand elongated from derivatized oligo. Although any nucleic acid containing the sequence complementary to the capture oligo will be retained on the membrane, only PCR products containing the derivatized oligonucleotide primer can bind the modified BAP-his_6 enzyme. The PCR product is detected by BAP activity retained on the membrane after adequate washing procedure. This setup allows PCR with crude lysates, since contaminating agents can be removed by filtration and only the PCR products retained by hybridization to the membrane bound oligonucleotide give rise to a detectable signal. This setup is also amenable to multiplexing (see above).

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the invention and are covered by the following claims.

We claim,

1. A composition comprised of at least two biopolymers conjugated to an insoluble support by at least one reversible linkage.

2. A composition according to claim 1, wherein the at least two biopolymers are comprised of nucleic acids.

3. A composition according to claim 1, wherein the at least two biopolymers are comprised of polypeptides.

4. A composition according to claim 1, wherein the at least two biopolymers are comprised of a nucleic acid and a protein.

5. A composition according to claim 1, wherein the at least one reversible linkage is formed through a trityl derivative, a chelate complex, a hydrophobic interaction or a photocleavable functionality.

6. A composition according to claim 1, wherein the insoluble support is selected from the group consisting of: a flat surface, a comb and a bead.

7. A composition according to claim 6, wherein the insoluble support is selected from the group consisting of: a silicon wafer, glass plate, metal, plastic, film and composites thereof with pits or wells.

8. A composition according to claim 7, wherein the biopolymer is conjugated to the insoluble support in an array format.

9. A composition according to claim 7, wherein the bead is comprised of an inorganic material selected from the group consisting of: silica, Controlled Pore Glass (CPG), plastic, metal, cellulose, Sepharose and Sephadex.

10. A composition according to claim 6, wherein the insoluble support is comprised of a magnetic or electromagnetic material.

11. A composition according to claim 2, wherein the nucleic acid is selected from the group consisting of: deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or analogs or mimetics of DNA or RNA.

12. A compostion according to claim 3, wherein the polypeptide is selected from the group consisting of an antibody, enzyme, receptor or peptide.

13. A composition according to claim 1, which contains a spacer between the biopolymer and the insoluble support.

14. A composition according to claim 4, which is made by the formation of a chelate complex between the nucleic acid and the polypeptide.

15. A composition according to claim 14, wherein the chelate complex is formed by the reaction of a nucleic acid containing a chelate functionality with a polypeptide containing an imidazoyl functionality in the presence of a metal ion.

16. A composition of claim 14, wherein the chelate complex is formed by the reaction of a nucleic acid containing an imidazoyl functionality with a polypeptide containing a chelate functionality in the presence of a metal ion.

17. A composition according to claim 15 or 16, wherein the polypeptide is an enzyme.

18. A composition according to claim 17, wherein the enzyme is an alkaline phosphatase.

19. A method according to claim 18, wherein the enzyme is bacterial

alkaline phosphatase (BAP).

20. A method for making a composition of claim 1, comprising the steps of:

a) immobilizing a nucleic acid to an insoluble support via a first reversible linkage; and

b) conjugating said nucleic acid with a polypeptide via a second reversible linkage.

21. A method according to claim 20, wherein the first or second reversible linkage is formed through a trityl derivative, a chelate complex, a hydrophobic interaction or a photocleavable functionality.

22. A method according to claim 20, wherein step b), the first or second reversible linkage forms a chelate complex.

23. A method according to claim 22, wherein the first or second reversible linkage is formed by the reaction of a nucleic acid containing a chelate functionality with a polypeptide containing an imidazoyl functionality in the presence of a metal ion.

24. A method according to claim 22, wherein the first or second reversible linkage is formed by the reaction of a nucleic acid containing an imidazoyl functionality with a polypeptide containing a chelate functionality in the presence of a metal ion.

25. A method according to claim 20, wherein the first or second reversible linkage are formed from functionalities or precursors, which are introduced into the nucleic acid during enzymatic synthesis.

26. A method according to claim 25, wherein the enzymatic synthesis

is part of an amplification procedure.

27. A method of claim 26, wherein the amplification procedure is selected from the group consisting of the polymerase chain reaction (PCR), the ligase chain reaction (LCR) and strand displacement amplification (SDA)..

28. A method according to claim 25, wherein the enzymatic synthesis is part of a nucleic acid sequencing procedure.

29. An oligonucleotide analog comprised of a β-cyanoethylphosphoamidite functionality with a chelate functionality.

30. An oligonucleotide analog of claim 29, wherein the chelate functionality is a precursor of nitrilotriacetic acid derived from either serine, cysteine or lysine.

31. An oligonucleotide analog comprised of a heterobifunctional trityl group with a chelate functionality.

32. An oligonucleotide analog of claim 31, wherein the chelate functionality is a precursor of nitrilotrisacetic acid derived from serine, cysteine or lysine.

33. An oligonucleotide analog comprised of a β-cyanoethylphosphoamidite functionality with an imidazolyl functionality.

34. An oligonucleotide analog comprised of a heterobifunctional trityl group with a oligohistidyl or oligoimidazolyl sequence.

35. An oligonucleotide analog according to claim 34, wherein the oligohistidyl sequence is present at the 5'- or 3'- terminus.

36. An oligonucleotide analog comprised of an imidazolylnucleoside-β-cyanoethylphosphoamidite.

37. A member selected from the group consisting of: nucleoside triphosphates, 2'-deoxynucleoside triphosphates, 3'-deoxynucleoside triphosphates and 2',3'-dideoxynucleoside triphosphates, wherein the member contains a chelate functionality at either C5 in the pyrimidine ring of thymine, uracil, or cytidine or at C8 in the purine ring of adenine, guanine or hypoxanthine.

38. A member selected from the group consisting of: nucleoside triphosphates, 2'-deoxynucleoside triphosphates, 3'-deoxynucleoside triphosphates and 2',3'-dideoxynucleoside triphosphates, wherein the member contains an oligohistidyl or oligoimidazolyl chain at either C5 in the pyrimidine ring of thymine, uracil, or cytidine or at C8 in the purine ring of adenine, guanine or hypoxanthine.

39. A recombinant protein which carries at its C-terminus an oligopeptide chain, which is capable of forming a chelate complex in the presence of metal ions.

40. A recombinant protein according to claim 39 which has enzymatic activity.

41. A recombinant according to claim 40, which is an alkaline phosphatase, which has an alanine residue at its N-terminus instead of arginine-threonine and which has at its C-terminus a chain of six histidine residues.

42. A peptide which carries at its N- or C- terminus an oligohistidyl sequence, which is capable of forming a chelate complex in the presence of metal ions.

43. A peptide which carries at its N- or C- terminus a chelator functionality which is capable of forming a chelate complex in the presence of metal ions.

44. A composition of claim 1, wherein the insoluble support is linked via a spacer to the nucleic acid through a reversible heterobifunctional trityl group and the nucleic acid is conjugated to an enzyme through a reversible chelate functionality.

45. A scomposition according to claim 44 in which the polymer support is comprised of magnetic beads, the chelate complex is formed via the nitrilotriacetic acid functionality in the presence of Ni^{2+} and the enzyme is BAP-his_6.

46. A composition according to claim 44 in which the polymer support is a silicon wafer carrying the reversible functionalities to bind the nucleic acid either directly on the surface or through beads in pits or wells in an array format, the chelate complex is formed via nitrilotrisacetic acid functionality in the presence of Ni^{2+} and the enzyme is BAP-his_6.

47. A composition according to claim 44 in which the polymer support is the filter bottom in the wells of a microtiter filter plate, the chelate complex is formed via nitrilotrisacetic acid functionality in the presence of Ni^{2+} and the enzyme if BAP-his_6.

48. A method of using the composition according to claim 44 to purify and to detect products of nucleic acid amplification procedures.

49. A method of claim 48, wherein the amplification procedure is selected from the group consisting of: the polymerase chain reaction, the ligase chain reaction and strand displacement amplification.

50 A method for using the composition according to claim 44 for determining the sequence of a nucleic acid.

51. A method for using the composition according to claim 44 to purify and to detect the identity and relative quantity of mRNAs or their corresponding

cDNAs for genetic or expression profiling.

52. A method for using the composition according to claim 44 to purify and to detect products of nucleic acid amplification procedures.

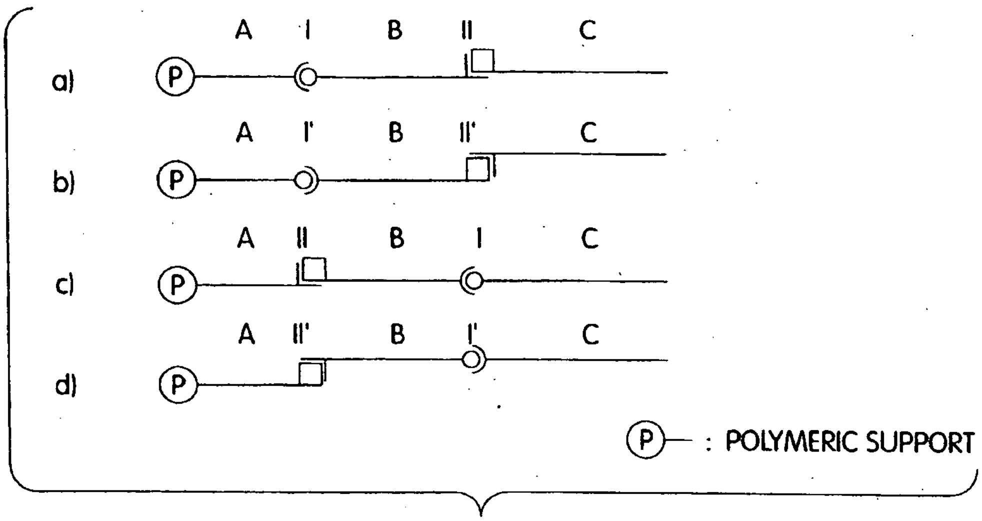


Fig. 1

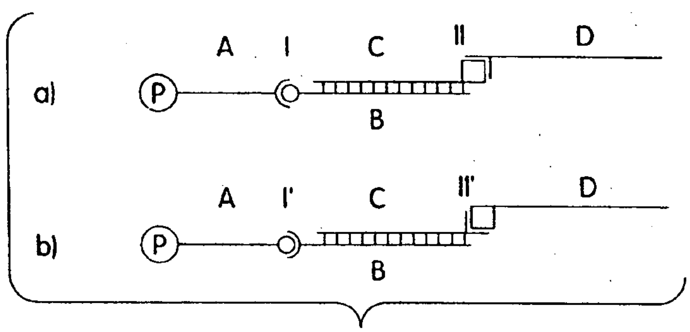


Fig. 2

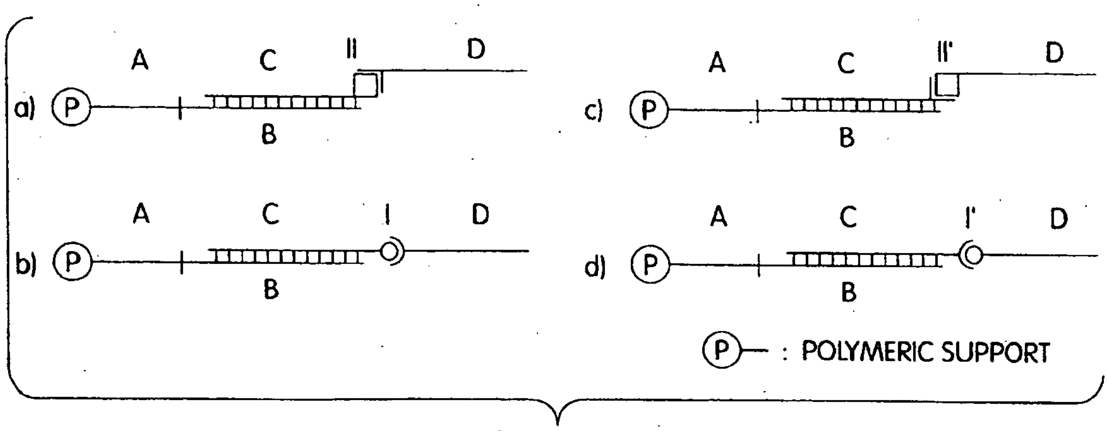
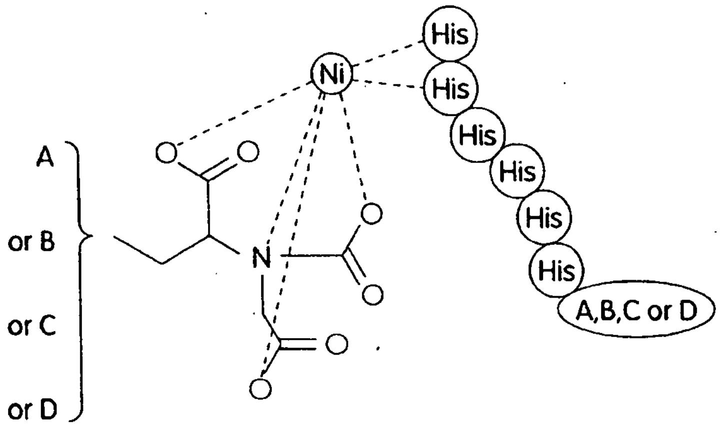


Fig. 3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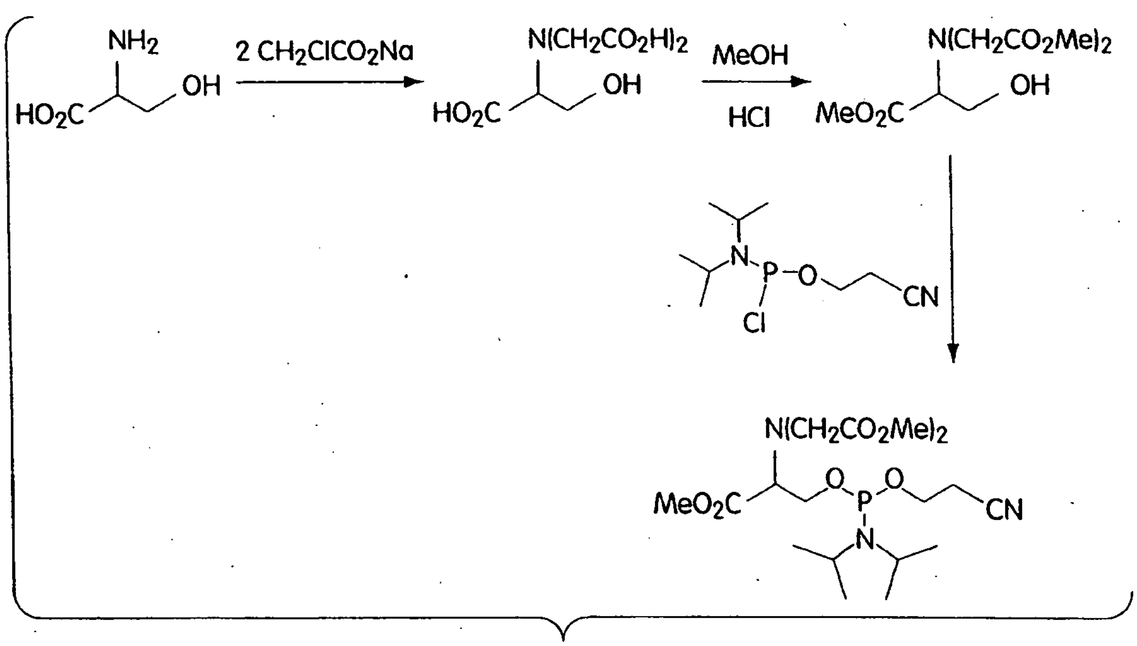
His
His
His
His
His
A,B,C or D
Ni
A
or B
or C
or D
N

Fig. 4


NH2
OH
HO2C
2 CH2ClCO2Na
N(CH2CO2H)2
OH
HO2C
MeOH
HCl
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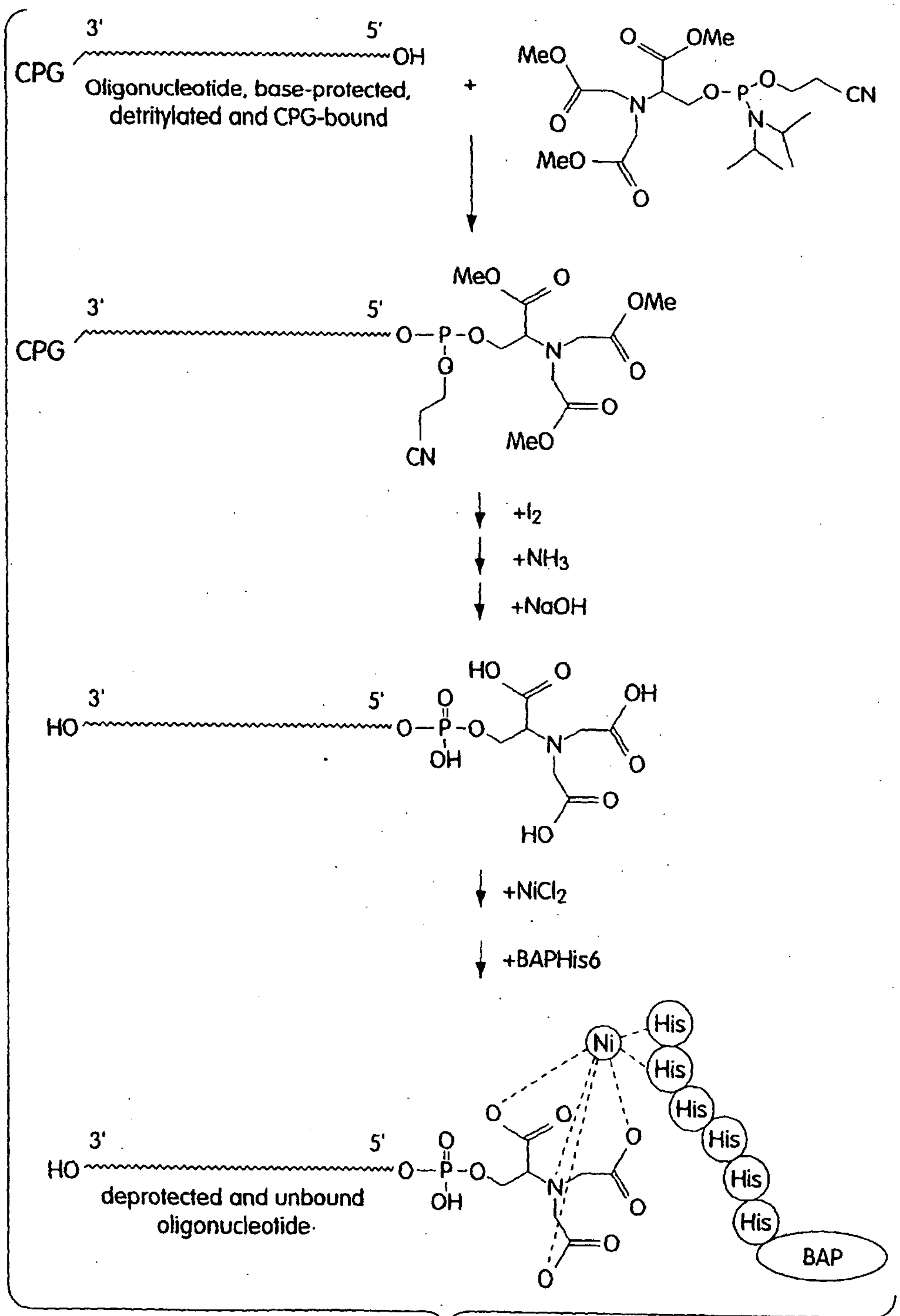
OH
MeO2C
N
P
O
CN
Cl
N(CH2CO2Me)2
MeO2C
O
P
O
CN
N

Fig. 5


3'
5'
CPG
OH
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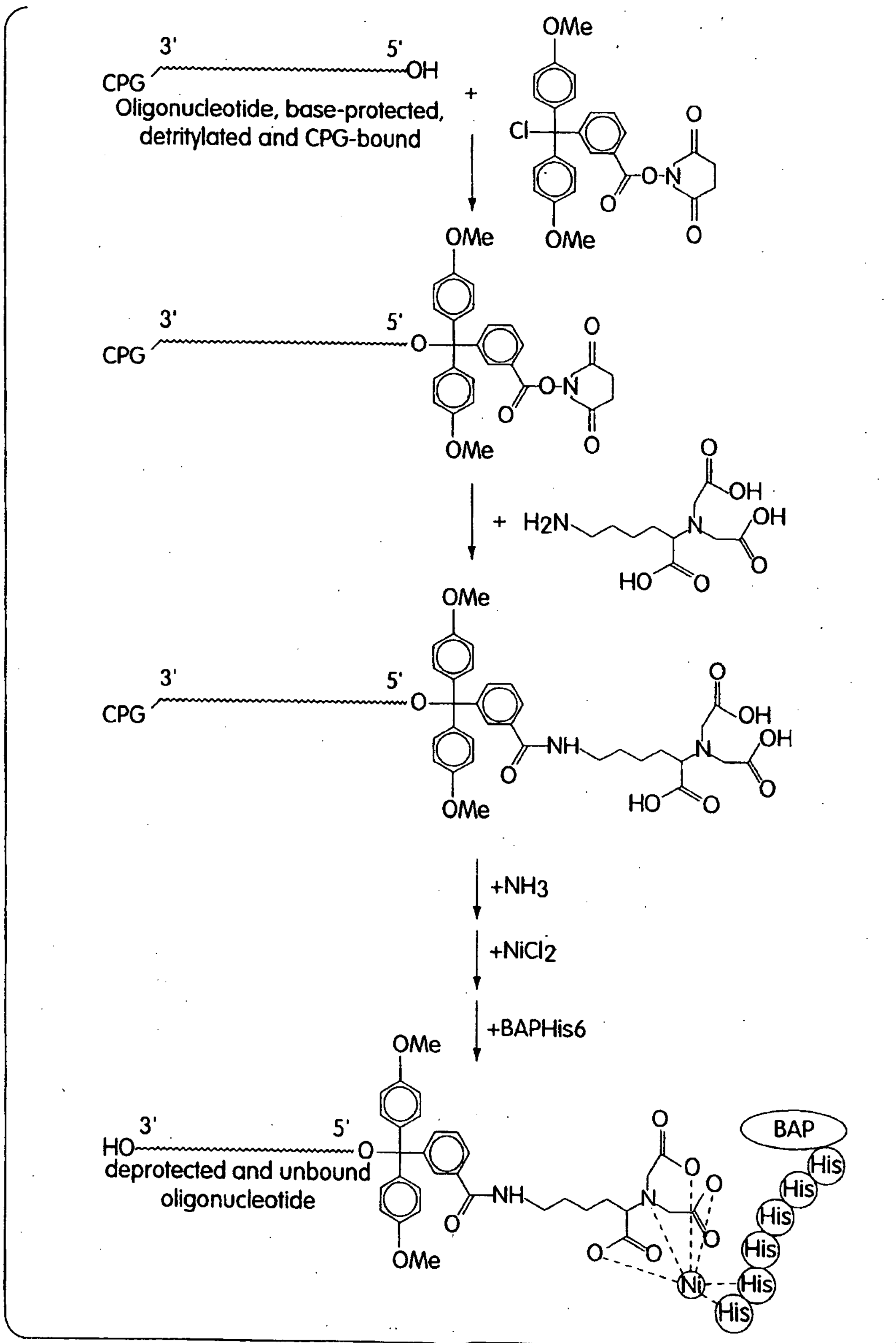
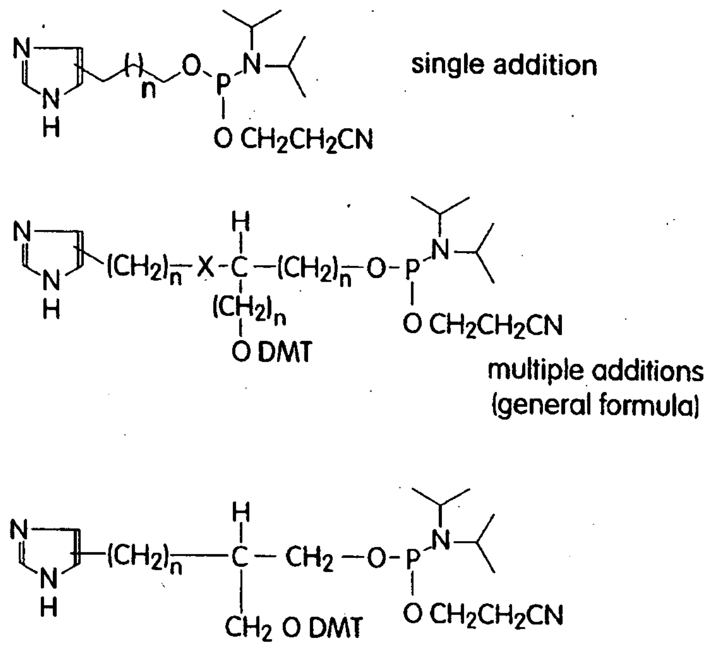
+
MeO
O
OMe
N
O
P
CN
MeO
+I2
+NH3
+NaOH
HO
OH
+NiCl2
+BAPHis6
Ni
His
BAP
deprotected and unbound oligonucleotide

Fig. 6



CPG
3'
5'
OH
Oligonucleotide, base-protected, detritylated and CPG-bound
+
OMe
Cl
O
O-N
OMe
+ H2N
N
OH
HO
+NH3
+NiCl2
+BAPHis6
HO
deprotected and unbound oligonucleotide
NH
Ni
His
BAP

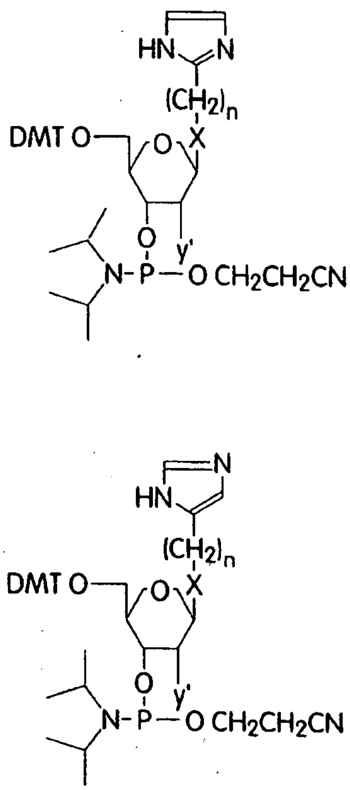
Fig. 7



X : - CH_2 - , - S - , - O - , - NH - , - NR - , - C(=O) - NH - , - C(=O) - O -

n : 0 - 20

Fig. 8



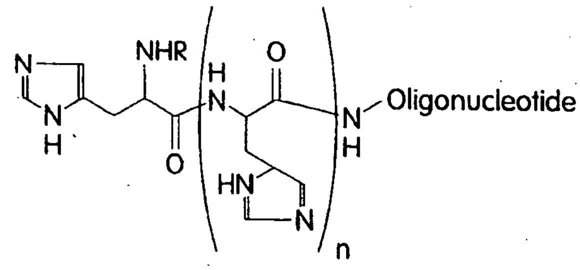
$X: -S-, -O-, -NH-, -NR-, -\overset{\overset{O}{\|}}{C}-NH-, -\overset{\overset{O}{\|}}{C}-O-$

n : 0 - 20

y' : - OR , - NHR , - SR , - F , - Cl , - Br , - O Me

R : Protecting Group (Lower alkyl, silyl alkyl, THP, etc.

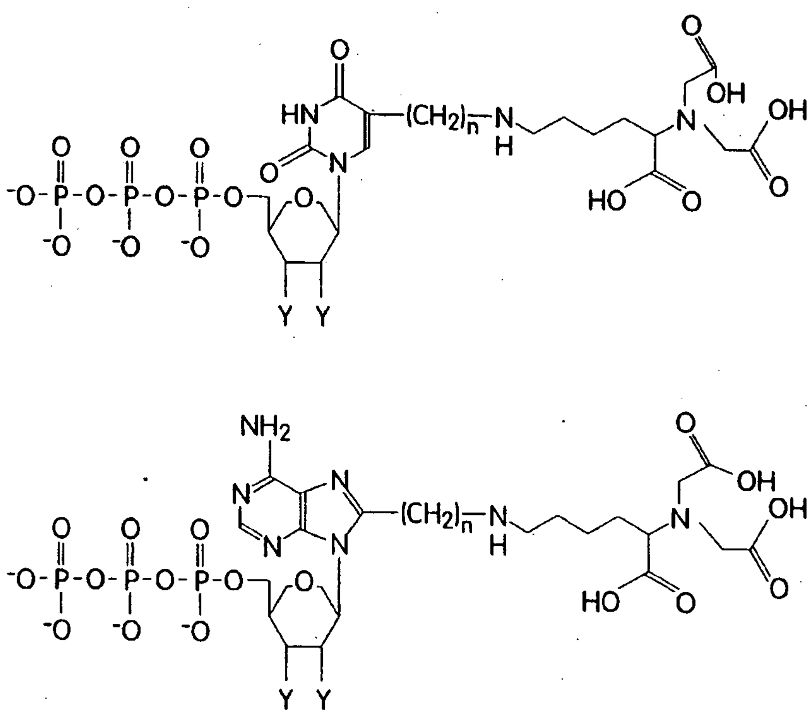
Fig. 9



R : Protecting Group (e.g. Fmoc , tBoc)

n : 0 - 20

Fig. 10



y : H ; OH

n : 0 - 20

Fig. 11

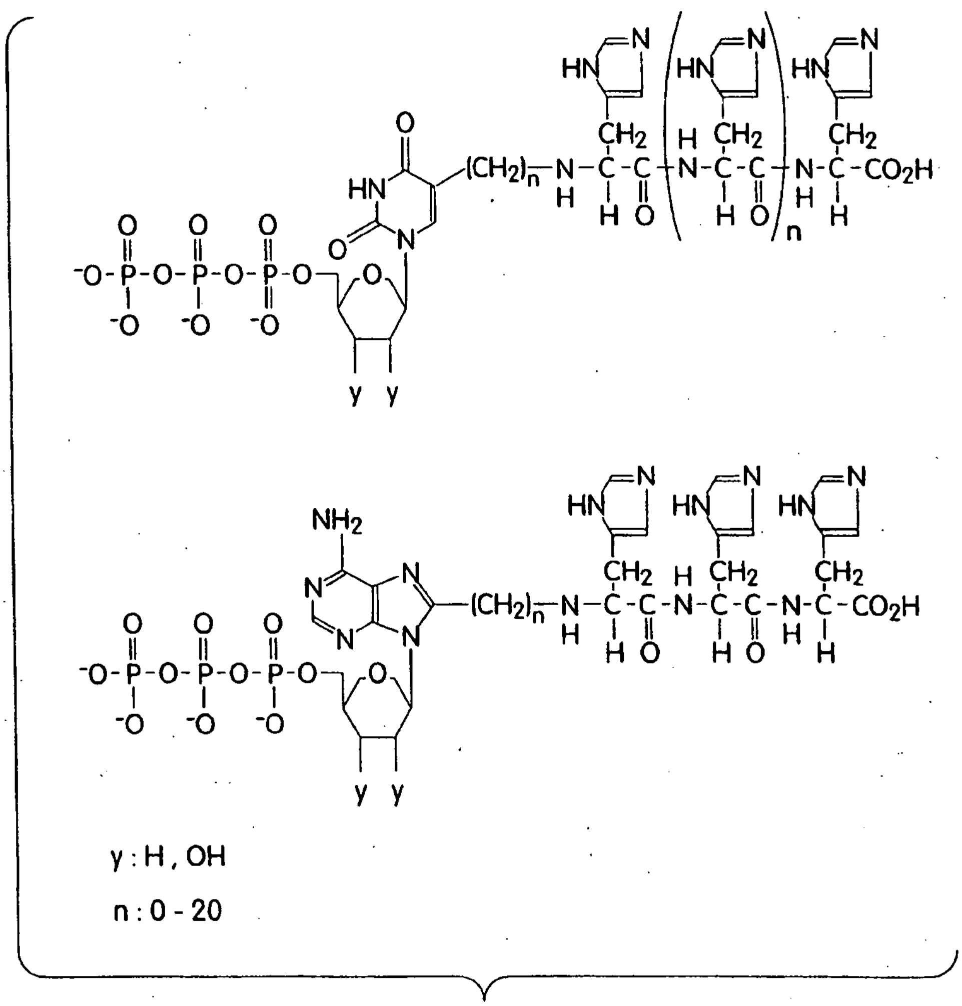


Fig. 12

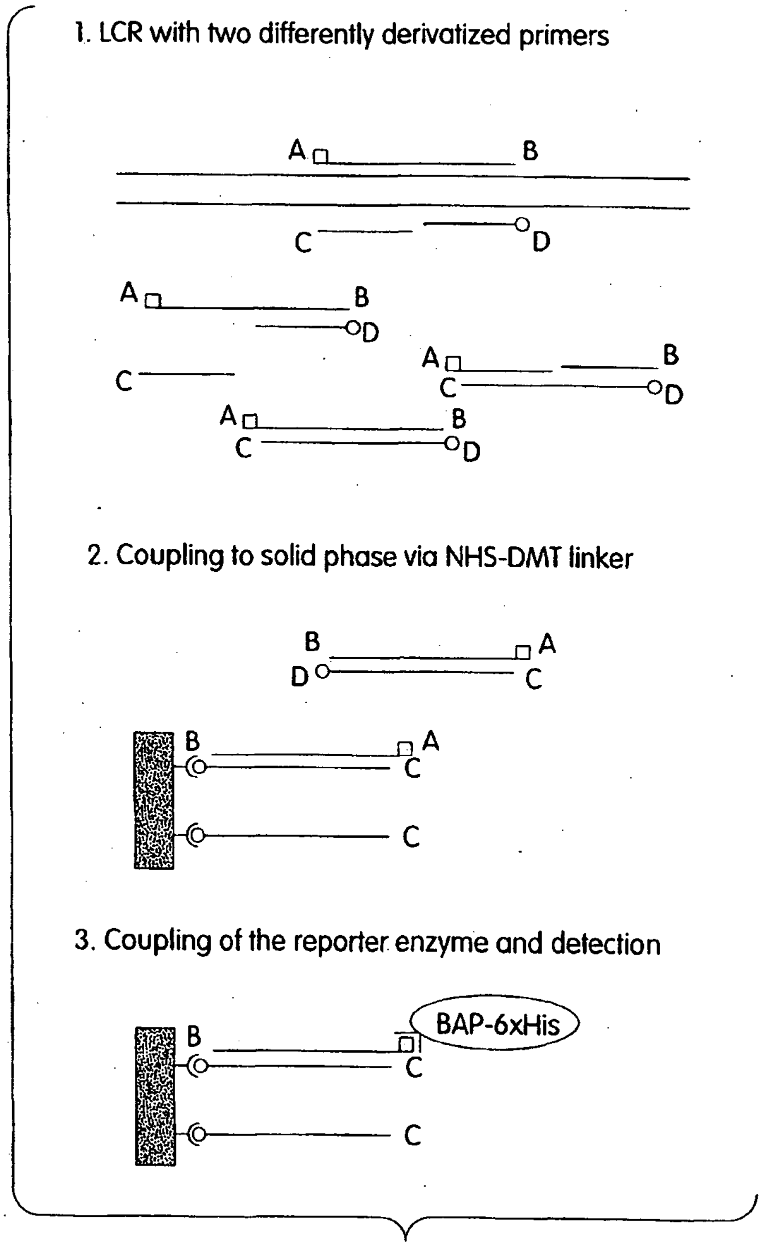

1. LCR with two differently derivatized primers
A
B
C
D
2. Coupling to solid phase via NHS-DMT linker
3. Coupling of the reporter enzyme and detection
BAP-6xHis

Fig. 13

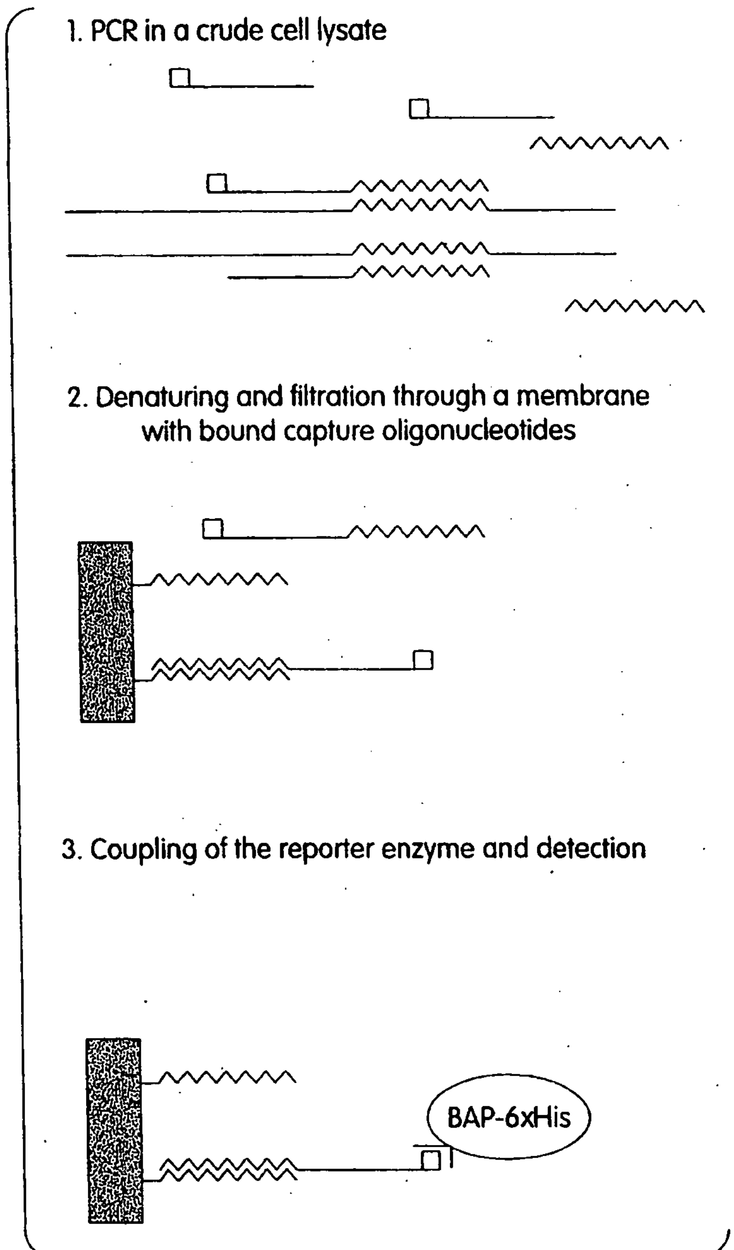

1. PCR in a crude cell lysate
2. Denaturing and filtration through a membrane with bound capture oligonucleotides
3. Coupling of the reporter enzyme and detection
BAP-6xHis

Fig. 14

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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G01N 33/543, C12N 11/00

A3

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(54) Title: A REVERSIBLE STOICHIOMETRIC PROCESS FOR CONJUGATING BIOMOLECULES

(57) Abstract

Compositions comprised of at least two biopolymers (e.g., nucleic acids or polypeptides), which are conjugated to an insoluble support by two different reversible linkages, which are cleavable under selective conditions, as well as methods and components for producing the same are described.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/02007

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 C12Q1/68 A61K47/00 G01N33/543 C12N11/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C12Q A61K G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 89 05616 A (BIO METRIC SYSTEMS INC) 29 June 1989 see the whole document ---	1,2, 5-10,13
X	US 5 410 068 A (COULL JAMES M ET AL) 25 April 1995 see the whole document ---	1,2, 5-10,13
X	US 5 582 981 A (TOOLE JOHN J ET AL) 10 December 1996 see abstract; claim 1 ---	1,2,5-9
X	WO 85 04674 A (LIFE TECHNOLOGIES INC) 24 October 1985 see claims 1-33 --- -/--	1,2,13

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[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 October 1998	07. 01. 99

Name and mailing address of the ISA	Authorized officer
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1

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90 01564 A (MICROPROBE CORP) 22 February 1990 see page 1-84	1,2,5-9, 13
A	ARSHADY E: "BEADED POLYMER SUPPORTS AND GELS II. PHYSICO-CHEMICAL CRITERIA AND FUNCTIONALIZATION" JOURNAL OF CHROMATOGRAPHY, vol. 586, no. 2, 22 November 1991, pages 199-219, XP000247969 see the whole document	6-9
A	PON R T ET AL: "DERIVATIZATION OF CONTROLLED PORE GLASS BEADS FOR SOLID PHASE OLIGONUCLEOTIDE SYNTHESIS" BIOTECHNIQUES, vol. 6, no. 8, 1 January 1988, pages 768-770, 773 - 775, XP000562920 see the whole document	1,2, 5-10,13
A	US 5 547 835 A (KOESTER HUBERT) 20 August 1996	1,2,13
A	DE 36 44 346 A (SAEULENTECHNIK DR ING HERBERT) 21 May 1987 see the whole document	1,2, 5-10,13
A	SCOUTEN W H ET AL: "REVERSIBLE IMMOBILIZATION OF ANTIBODIES ON MAGNETIC BEADS" ANALYTICAL BIOCHEMISTRY, vol. 205, no. 2, 1 September 1992, pages 313-318, XP000296795 see the whole document	1,5-10, 13
A	P.D.GERSHON ET AL.: "Stable Chelating Linkage for Reversible Immobilization of Oligohistidine Tagged Proteins in the BIAcore Surface Plasmon Resonance Detector." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 183, 1995, pages 65-76, XP002081778	1
E	WO 98 20020 A (KOSTER HUBERT ;LITTLE DANIEL P (US); SEQUENOM INC (US): CANTOR CHA) 14 May 1998 see claims 1-121	1,2, 5-10,13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/02007

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

2,11(completely); 1,5-10, 13(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 2,11 completely; 1,5-10,13 partially

   Composition comprising at least two biopolymers conjugated to an insoluble support by at least one reversible linkage. Wherein the at least two biopolymers are comprised of nucleic acids.

2. Claims: 3,12 completely; 1,5-10,13 partially

   Composition comprising at least two biopolymers conjugated to an insoluble support by at least one reversible linkage. Wherein the at least two biopolymers are comprised of polypeptides.

3. Claims: 4,14-28 completely; 1,5-10,13 partially

   Composition comprising at least two biopolymers conjugated to an insoluble support by at least one reversible linkage. Wherein the at least two biopolymers are comprised of nucleic acid and a protein.

4. Claims: 1,5-10,13 partially

   Composition comprising at least two biopolymers conjugated to an insoluble support by at least one reversible linkage. Wherein the at least two biopolymers are not comprised of two nucleic acids, two polypeptides, or a nucleic acid and a protein.

5. Claims: 29,30,33,36

   Oligonucleotides comprised of a B-cyanoethylphosphoramidite.

6. Claims: 31,32,34,35

   Oligonucleotides comprised of a heterobifunctional trityl group.

7. Claims: 37,38

   Nucleoside triphosphates with a chelate functionality on the base moiety

8. Claims: 39-41

   Recombinant proteins with enzymatic activity.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

9. Claims: 42,43

A peptide with a chelator at the N or C terminus.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/02007

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 8905616	A	29-06-1989	AT	137104	T	15-05-1996
			CA	1335721	A	30-05-1995
			DE	3855238	D	30-05-1996
			DE	3855238	T	14-11-1996
			DK	152590	A	24-08-1990
			EP	0407390	A	16-01-1991
			JP	3503005	T	11-07-1991
			NO	180657	B	10-02-1997
			US	5512329	A	30-04-1996
			US	5002582	A	26-03-1991
			US	5741551	A	21-04-1998
			US	5258041	A	02-11-1993
US 5410068	A	25-04-1995	DE	69015566	D	09-02-1995
			DE	69015566	T	10-08-1995
			EP	0424819	A	02-05-1991
			JP	3279371	A	10-12-1991
US 5582981	A	10-12-1996	AU	1435492	A	15-09-1992
			AU	1456092	A	15-09-1992
			CA	2104698	A	22-08-1992
			EP	0572529	A	08-12-1993
			JP	6508022	T	14-09-1994
			WO	9214842	A	03-09-1992
			WO	9214843	A	03-09-1992
WO 8504674	A	24-10-1985	EP	0177497	A	16-04-1986
			JP	61501746	T	21-08-1986
WO 9001564	A	22-02-1990	NONE			
US 5547835	A	20-08-1996	US	5605798	A	25-02-1997
			US	5691141	A	25-11-1997
			AU	694940	B	06-08-1998
			AU	5992994	A	15-08-1994
			CA	2153387	A	21-07-1994
			EP	0679196	A	02-11-1995
			JP	8509857	T	22-10-1996
			WO	9416101	A	21-07-1994
DE 3644346	A	21-05-1987	NONE			
WO 9820020	A	14-05-1998	AU	5106998	A	29-05-1998
			AU	5198098	A	29-05-1998
			WO	9820166	A	14-05-1998
			AU	5247298	A	29-05-1998
			WO	9820019	A	14-05-1998

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(54) Title: DIAGNOSIS OF DISEASE STATE USING MRNA PROFILES

(57) Abstract

Disclosed are diagnostic techniques for the detection of a human diseased state. Genetic probes and methods useful in monitoring the progression and diagnosis of the disease state are described. The invention relates particularly to probes and methods for evaluating the presence of RNA species that are differentially expressed in the peripheral blood of individuals with the disease state compared to normal healthy individuals. Further disclosed is a multivariate diagnostic model for prostate cancer in a population of men with moderately elevated total serum PSA ($\geq$ 2.0 ng/ml). Results of quantitative serum assays for the UC325 gene product [Interleukin-8 (IL-8)], total prostate specific antigen (t-PSA), as well as Free/Total (f/t PSA) ratios were combined to enhance the sensitivity of prostate cancer diagnosis in a defined urologic population diagnosed either organ-confined prostate cancer (clinical stage A & B), non-organ-confined prostate cancer (clinical stage C or D) or benign prostatic hyperplasia (BPH). The additional ability of UC325 gene product serum levels to accurately stage prostate cancer independently of t-PSA of f/t PSA is disclosed.

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arbitrary oligonucleotides. PCR amplification products were labeled using α-32P-dCTP and were visualized by autoradiography after electrophoresis on denaturing polyacrylamide gels. A number of bands appeared to be differentially expressed, and were cloned as described above.

UC Band #321 was confirmed by RT-PCR to be down regulated in the peripheral blood of prostate cancer patients, with a four-fold decrease observed compared with normal individuals. The DNA sequence of Band #321 does not match any known sequences in the GenBank database. It therefore represents a previously undescribed gene product.

UC Band #302 and UC Band #325 were both observed to be up regulated in the peripheral blood of metastatic prostate cancer patients. UC Band #302 is identical in sequence to a portion of the sequence of elongation factor 1-α (GenBank Accession #X03558). This band was modestly increased between 1.6 and 2-fold in metastatic cancer patients compared with normal individuals.

UC Band #325 was found to consist of two different alternatively spliced forms of mRNA, encoded by the interleukin-8 (IL-8) gene. UC Band #325-1, the previously identified mRNA species of IL-8 (Genbank Accession #Y00787), is approximately seven-fold more abundant in the peripheral blood of metastatic prostate cancer patients. The alternatively spliced IL-8 mRNA, containing intron #3 of the IL-8 gene (Genbank Accession #M28130) is up to seven-fold less abundant in the peripheral blood of metastatic prostate cancer patients. Fig. 1A shows relative quantitative RT-PCR of the differential expression of IL-8 (=UC235) in peripheral blood of patients with metastatic prostate cancer (M) and normal individuals (N) at different PCR cycles (cy). The two alternatively spliced forms of the IL-8 mRNA are observed. The upper band (int.+) includes intron 3 in the mature mRNA. The lower band (int.-) lacks intron 3. Fig. 1B shows relative quantitative RT-PCR showing Differential Expression of IL-8 (UC325) in peripheral blood of patients with metastatic prostate cancer in lanes 1-5 and a pool of normal individuals (N). The alternatively spliced forms of the IL-8 mRNA observed are different between normal individuals and those with prostate cancer. Overall, there is an approximately 30-fold change in the ratios of the two spliced forms of IL-8 mRNA in individuals with metastatic prostate cancer compared with normal individuals. These results have been confirmed by relative quantitative RT-PCR.

As described above, an increased expression of IL-8 mRNA has been previously reported in cancer patients. However, this represents the first finding of an alternatively spliced form of IL-8 mRNA, containing intron 3, that is significantly more abundant in normal individuals compared

with metastatic prostate cancer patients. These results are surprising in view of previous reports which had failed to find any alternatively spliced forms of IL-8 mRNA in normal individuals or cancer patients.

It will be recognized that the genes and gene products (RNAs and proteins) for the above described markers of metastatic prostate cancer are included within the scope of the disclosure herein described. It will also be recognized that the diagnosis and prognosis of metastatic prostatic cancer by detection of the nucleic acid products of these genes are included within the scope of the present invention. Serological and other assays to detect these mRNA species or their translation products are also indicated. It is obvious that these assays are of utility in diagnosing metastatic cancers derived from prostate and other tissues.

Most significantly, these Examples demonstrate the feasibility of using RNA fingerprinting to identify mRNA species that are differentially expressed in the peripheral blood of patients with asymptomatic diseases or in patients with symptoms that are insufficient for a definitive diagnosis. It will be appreciated that this technique is applicable not only to the detection and diagnosis of prostate and other cancers, but also to any other disease states which produce significant effects on lymphocyte gene expression. Uses which are contemplated within the scope of the present disclosure include the detection and diagnosis of clinically significant diseases that requires medical intervention, including but not limited to asthma, lupus erythromatosis, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, autoimmune thyroiditis, ALS, interstitial cystitis and prostatitis.

TABLE 2

Genes Whose mRNAs have Abundances that Vary in Metastatic Prostate Cancer Relative to Normal Individuals

Name of cDNA Fragment	Sequence Determined	Confirmed by RT-PCR	Previously Known
UCPB 35	Yes	Yes	GB #T03013
UC 302 SEQ ID NO:3	Yes	Yes	EF 1-α
UC 321 SEQ ID NO:2	Yes	Yes	No
UC 325-1 SEQ ID NO:4	Yes	Yes	GB #Y00787
UC 325-2 SEQ ID NO:5	Yes	Yes	IL-8

TABLE 3.

Oligonucleotides used in the relative quantitative RT-PCR portion of these studies.

Oligonucleotides used to examine the expression of genes:

UCPB Band #35 (previously uncharacterized gene).

5' TGCAAACTTTCACCTGGACTT3', SEQ ID NO:10

5' CTTGTGACTTGCTTTGATAGAATG3', SEQ ID NO:11

UC Band #302 (elongation factor 1-α).

5' GACAACATGCTGGAGCCAAGTGC3', SEQ ID NO:12

5' ACCACCAATTTTGTAAGAACATCCT3', SEQ ID NO:13

UC Band #321 (previously uncharacterized gene).

5' TGTCCAGAGATCCAAGTGCAGAAGG3', SEQ ID NO:14

5' GAGCTCCAGGAGACAGAAGCCATAG3', SEQ ID NO:15

UC Band #325-1 (IL-8).

5' GGGCCCCAAGGAAAACT 3', SEQ ID NO:16

5' TGGCAACCCTACAACAGACC3', SEQ ID NO:17

UC Band #325-2 (IL-8).

5' GGGCCCCAAGGAAAACT3', SEQ ID NO:18

5' TGGCAACCCTACAACAGACC3', SEQ ID NO:19

Controls used to normalize relative quantitative RT-PCR

ß-actin

5' CGAGCTGCCTGACGGCCAGGTCATC3', SEQ ID NO:8

5' GAAGCATTTGCGGTGGACGATGGAG3', SEQ ID NO:9

Asparagine Synthetase (AS)

5' ACATTGAAGCACTCCGCGAC3', SEQ ID NO:20

5' AGAGTGGCAGCAACCAAGCT3', SEQ ID NO:21

Example 4:

DNA Sequences of Markers of Metastatic Prostate Cancer

The DNA sequences of the markers of metastatic prostate cancer were determined by Sanger dideoxy sequencing as detailed above. The identified sequences are provided in Table 4.

TABLE 4.

DNA Sequences of Markers of Metastatic Prostate Cancer:

UCPB Band #35 (SEQ ID NO:1) Matches a fetal brain EST, GenBank Accession # T03013

5'GGCAGGGGCTTGTGACTCTAAGATGGCTTCATTCACATGCCTAGGGCCTCAGTAGG
ATGACTGGCATGGCCCTGGAAAACTGCGAAGTCTTCTCTCTGTGCAAACTTTCACCT
GGACTTTTTATATGATTCTGGAAGTATTCCAAGAAGGCAAAAGTAAAAACTGCAAA
GCGTCTTAAAATAGAAGTTCAGAAGCCACATTATATCACTTCTGTTGCATTCTATCA
AAGCAAGTCACAAGCCCCTGCCAATCA 3'

UC Band # 321 (SEQ ID NO:2) previously uncharacterized Gene

5'CACACACTCCCCCATTCTGAGCCCCAAGAGGCTCATCCCTAAGGATGTCCAGAGA
TCCAAGTGCAGAAGGAGAATGTGGTGAGGCTATTTATTCCCCCAGTGCCTTCCCTGC
TGGGCTATGGATGAACAGTGGCTGACTTCATCTAGGAAAGAGCTATGGCTTCTGTCT
CCTGGAGCTCACCA 3'

UC Band # 302 (SEQ ID NO:3) Human Elongation Factor 1-alpha, GenBank Accession #X03558

5'GGTGAGCCCCAGGAGACAGAAGAGATATGAGGAAATTGTTAAGGAAGTCAGCAC
TTACATTAAGAAAATTGGCTACAACCCCGACACAGTAGCATTTGTGCCAATTTCTGG
TTGGAATGGTGACAACATGCTGGAGCCAAGTGCTAACATGCCTTGGTTCAAGGGAT
GGAAAGTCACCCGTAAGGATGGCAATGCCAGTGGAACCACGCTGCTTGAGGCTCTG
GACTGCATCCTACCACCAACTCGTCCAACTGACAAGCCCTTGCGCCTGCCTCTCCAA
GGATGTTCTTACAAAATTGGTGGTATTGGTACTGTTCCCTGTTTGGCCGAATTGGAA
AACTGGTGTTCCTCCAAACCCCGGTTATGGTGGGTTTCCTCCTCCTTGGA 3'

UC Band #325-1 (SEQ ID NO:4) Human IL-8 mRNA, GenBank Accession #Y00787

5'GGGCGGAACAAGGGAGCGCTAAAAGGAAATTAGGATGTCAGGTGCATAAAGGAC
ATAATTCCAAAACCTTTCCAAACCCCAAATTTATTCAAAGGAACTGAGGAGTGGATT
GAGGAGTGGACCAACACTGGCGCCAAACACAGAAATTATTGTAAAGCTTTCTGATG

GAAGAGAGCTCTGTCTGGGCCCCAAGGAAAACTGGGTGCAGAGGGTTGTGGAGAAG TTTTTGAAGAGGGCTGAGAATTCATAAAAAAATTCATTCTCTGTGGTATCCAAGAAT CAGTGAAGATGCCAGTGAAACTTCAAGCAAATCTACTTCAACACTTCATGTATTGTG TGGGTCTGTTGTAGGGTTGCCAGTTGTT 3'

UC Band #325-2 (SEQ ID NO:5) Human IL-8 mRNA Containing Intron #3

5'GCTTGGGCCCCAAGGAAAACTGGGTGCAGAGGGTTGTGGAGAAGTTTTTGAAGAG GTAAGTTATATATTTTTGAATTTAAAATTTGTCATTTATCCGTGAGACATATAATCCA AAGTCAGCCTATAAATTTCTTTCTGTTGCTAAAAATCGTCATTAGGTATCTGCCTTTT TGGTTAAAAAAAAAAGGAATAGCATCAATAGTGAGTGTGTTGTACTCATGACCAGA AAGACCATACATAGTTTGCCCAGGAAATTCTGGGTTTAAGCTTGTGTCCTATACTCTT AGTAAAGTTCTTTGTCACTCCCAGTAGTGTCCTATGTTAGATGATAATGTCTTTGATC TCCCTATTTATAGTTGAGAATATAGAGCATGTCTAACACATGAATGTCAAAGACTAT ATTGACTTTTCAAGAACCCTACTTTCCTTCTTATTAAACATAGCTCATCTTTATATTGT GAATTTTATTTTAGGGCTGAGAATTCATAAAAAAATTCATTCTCTGTGGTATCCAAG AATCAGTGAAGATGCCAGTGAAACTTCAAGCAAATCTACTTCAACACTTCATGTATT GTGTGGGTCTGTTGTAGGGTTGCCA 3'

Example 5:

Detection and Differential Diagnosis of BPH versus Localized and Advanced Stage Prostate Carcinomas Using Combinations of IL-8 with Other Prostate Disease Markers.

A total of 164 serum specimens from normal men or men with a biopsy confirmed diagnosis of BPH or prostate cancer were studied. These serum specimens were provided by Dr. George Wright from the Virginia Prostate Center at the Eastern Virginia Medical School and by Dr. Robert Vessella from the University of Washington or were normal donors from UroCor, Inc. All patients were biopsy-confirmed for either BPH or prostate carcinoma (stages A, B, and C only) within six months after PSA serum collection and/or a DRE-positive diagnosis. All patient sera were obtained prior to any surgical or hormonal therapies. The mean age of the total sample was 69.4 ± 8.6 years (range = 37 - 91 years) old.

The subset of patients utilized for multivariate diagnostic serum model consisted of 13 BPH and 64 CaP (Stages A, B, C) cases from the parent population (Marley *et al.*, 1996). All patients in the subset had a total PSA between 2.0 - 20.0 ng/ml, which is a standard range for f/t

PSA testing (Marley *et al.*, 1996). Also evaluated were a subset of Stage D CaP patients, with t-PSA values ranging from 6.5 - 867 ng/ml.

Diagnosis	N	Mean Age ± Std. Dev. (Range)
Normal	8	< 50 years
BPH	55	66.4± 8.6 (37 - 87) years
CaP Stage A	24	74.7±7.8 (61 - 91) years
CaP Stage B	48	68.3±7.9 (51 - 85) years
CaP Stage C	14	68.9±6.9 (60 - 80) years
CaP Stage D	14	72.3±8.6 (58 - 86) years

Table 5 shows the distribution of the total PSA levels, the f/t PSA ratios, and the UC325 levels for the 164 patients, broken down by normals, BPH, and Stages A, B, C, & D prostate cancer. Only the BPH, Stage A, Stage B, and Stage C prostate cancer patients were included in the statistical analysis.

TABLE 5

UC325 Patient Sample Characteristics (n = 164)

		Mean Value ± Std. Dev.		
Diagnosis	**N**	**UC325 (pg/ml)**	**Total PSA (ng/ml)**	**f/t PSA Ratio (%)**
Normal	8	0.2 ± 0.6	N/A	N/A
BPH	55	6.8 ± 6.1	6.9 ± 4.0	21.9 ± 10.9%
CaP Stage A	24	19.1 ± 10.4	6.2 ±2.7	14.6 ± 10.5%
CaP Stage B	48	13.5 ± 9.5	8.8 ± 6.6	11.9 ± 5.7%
CaP Stage C	15	19.1 ± 7.9	16.2 ± 7.6	11.2 ± 8.3
CaP State D	14	78.9 ± 197	244 ± 332	12.4 ± 7.1%

Table 6 illustrates the ability for f/t PSA ratio at three different cutoffs to differentiate prostate cancer and BPH in the inventors' patient sample. UC325 (IL-8) and t-PSA are analyzed at single Classification and Regression Tree (CART) cutoff points for the same outcome. Note the significant improvement in both sensitivity and specificity contributed by the UC325 (IL-8) serum assay to detect clinically organ confined. The combination of UC325 (IL-8), treated as a continuous variable, and t-PSA or f/t PSA ratio provides a highly predictive multivariate test

system to diagnose CaP (clinical stages A & B) without any interference provided by BPH in the inventors' patient subset.

TABLE 6

Ability of Serum Tests to Discriminate BPH and CaP.

Serum Test	Cutoff	Sensitivity	Specificity	AUC	p-value
f/t PSA Ratio	11%	52.9%	91.9%	0.7905	< 0.0001
""	14%	70.1%	80.0	""	""
""	20%	85.1	47.3	""	""
UC325	9.8 pg/ml	72.4%	74.5%	0.7973	<0.0001
Total PSA	14.8 ng/ml	17.2%	98.2%	0.5995	0.0134
f/t PSA & UC325	0.69**	71.3%	90.9%	0.8784	<0.0001
Total PSA & UC325	0.64**	62.1%	85.5%	0.8069	<0.0001

*All cutoff's determined using Classification and Regression Tree Analysis (CART)

**Predicated Probability value calculated using logistic regression function

To further substantiate the results of Table 6, individual analysis using Receiver Operator Characteristic (ROC) curves are provided for each variable. Figure 2 illustrates the ability of t-PSA to distinguish BPH and Stages A, B, and C prostate cancer. Figure 3 shows the ability of f/t PSA ratio to distinguish BPH and Stages A, B, and C prostate cancer. Figure 4 shows the ability of UC325 (IL-8) alone to distinguish BPH and Stages A, B, and C prostate cancer. Figure 5 shows the ability of the combination of UC325 (IL-8) and total PSA (t-PSA) to distinguish BPH and Stages A, B and C prostate cancer. Figure 6 shows the ability of the combination of UC325 (IL-8) and the f/t PSA ratio to distinguish between BPH and stages A, B and C prostate cancer. It is apparent that the combination of UC325 measurement with either t-PSA or f/T PSA provides a significant increase in sensitivity of detection, while maintaining a high degree of specificity. Thus, the combination of UC325 (IL-8) with other prostate disease markers, such as t-PSA or f/t PSA ratio, provides a significant advance in the detection and differential diagnosis of prostate cancer.

Table 7 presents the correlation values for the different serum markers. This table clearly shows that the UC325 biomarker provides information which is independent of that provided by the f/t PSA ratio.

TABLE 7

Correlation Values for BPH vs Stages A, B & C (n = 142)

	Diagnosis	Total PSA (ng/ml)	f/t PSA Ratio (%)	UC325 (pg/ml)	Age	Clinical Stage
Diagnosis	1.0000	0.5647	-0.1912	0.2262	0.1590	0.3497
Total PSA (ng/ml)	0.5647	1.000	-0.2319	0.5991	0.0898	0.3729
f/t PSA Ratio (%)	-0.1912	0.2319	1.0000	-0.2142	0.0641	-0.4126
UC325 (pg/ml)	0.2262	0.5991	0.2142	1.0000	0.0881	0.2486
Age	0.1590	0.0898	0.0641	0.0881	1.0000	0.1372
Clinical Stage	0.3497	0.3729	-0.4126	0.2486	0.1372	1.0000

Table 8 clearly demonstrates a relationship between tumor burden and serum UC-325 gene product measured by IL-8 assay. Note that as biopsy-confirmed clinical stage of the cancer increases, so does the IL-8 serum marker concentration, whereas the same relationship did not occur with [t-PSA] or f/t PSA ratio.

TABLE 8

UC325 Culled Dataset, One High and Low Value Removed (n=164)

		UC325 (10 pm/ml Cutoff)		UC325 (15 pg/ml Cutoff)	
Specimen Stage	N	Negative	Positive	Negative	Positive
Normal	8	8 (100%)	0 (0%)	8 (100%)	0 (0%)
BPH	55	41 (75%)	14 (25%)	50 (91%)	5 (9%)
Stage A & B	72	25 (35%)	47 (65%)	43 (60%)	29 (40%)
Stage C	15	0 (0%)	15 (100%)	5 (33%)	10 (67%)
Stage D	14	2 (14%)	12 (86%)	3 (21%)	11 (79%)

Example 6:

Identification of Markers of Metastatic Prostate and Breast Cancer by Use of ***RNA fingerprinting by PCR primed with oligonucleotides of arbitrary sequence.***

RNA fingerprinting displays PCR™ amplified cDNA fragments that represent a sample of RNA species derived from a population of total cell RNAs. When displayed side by side, comparisons of similarly produced fingerprints representing RNA populations from cells of differing physiologic states identifies mRNA species whose relative abundances vary between the examined physiologic states. In this study, RNA fingerprinting identified two cDNA fragments derived from mRNA species that had higher steady state abundances in the peripheral blood leukocytes of patients with recurrent metastatic prostate cancer as compared to a group of healthy volunteers.

Eight ml of peripheral blood was collected from healthy volunteers, patients with clinically and biopsy confirmed BPH, localized and advanced metastatic prostate cancer, and from patients with advanced metastatic breast cancer. Metastatic prostate and breast cancer patients that had failed a primary therapy and had evidence of recurrence of disease were selected. The metastatic prostate cancer patients had high (≥ 50 ng/ml) serum concentrations of PSA. Circulating nucleated peripheral blood cells were separated from erythrocytes by centrifugation in Vacutainer© CPT™ tubes (Becton Dickinson and Company, Franklin Lakes, N J). Total RNA was prepared from isolated nucleated peripheral blood cells by lysis with RNA Stat-60™ (Tel-Test, Inc.; Friendswood, TX) following the instructions provided by the vendor. Contaminating genomic DNA was removed from the total RNAs by digestion with RNase free DNaseI (GIBCO-BRL, Gaithersburg, MD). For the PCR™ based applications of RNA fingerprinting and relative quantitative RT-PCR™, it is absolutely critical that the total RNA is completely free of genomic DNA. Typically, 5.0 to 10.0 μg of total RNA was digested with 20-40 units of RNase free DNaseI in 100-200 μl of reaction volume for 20 min at 37°C.

Following digestion, the total RNAs were extracted with phenol (pH=4.3, Amresco, Inc., Solon, OH) and ethanol precipitated. To confirm that the RNA was free of contaminating genomic DNA, 500 ng to 1.0 μg of each DNaseI treated RNA was resuspended in water. These were used as templates for PCR™ using oligonucleotide primers that anneal to exons 3 and 4 of

the gene encoding PSA (exon 3: 5′ GCCTCAGGCTGGGGCAGCATT 3′ SEQ ID NO:22, exon 4: 5′ GGTCACCTTCTGAGGGTGAACTTGC 3′ SEQ ID NO:23). These primers anneal to opposite strands of genomic DNA that flank the 145 bp intron 3 of the PSA gene. PCR™ was performed at 94°C for 1:15 min, followed by 40 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1:15 min, then a final extension of 72°C for 5:00 min. RNA was considered DNA-free if no PCR™ products could be visualized upon gel electrophoresis that co-migrated with the PSA gene positive control of known human genomic DNA. If PSA gene products were observed after PCR™, the RNA was redigested with DNaseI and analyzed again for contaminating genomic DNA. After it was confirmed that the RNAs were free of genomic DNA, 500 ng to 1.0 μg of RNA was electrophoresed on a 1.2% agarose Tris Acetate EDTA (TAE) to visualize the ribosomal RNAs (Fridell *et al.*, 1995). Only RNA preparations for which the 28S ribosomal RNA could be visualized were selected for further analysis by RNA fingerprinting and relative quantitative RT-PCR™.

RNA fingerprinting with arbitrarily chosen oligonucleotide primers (Welsh *et al.*, 1992) is conceptually similar to differential display (Liang and Pardee, 1992), except that oligonucleotides of arbitrary sequence are used to prime both strands of cDNA synthesis instead of just second strand synthesis, as in differential display. In this investigation, the strategy of RNA fingerprinting used was similar to that described in Ralph *et al.* (1993) except that oligonucleotide primers used were composed of two discrete domains. The 5′ domain of these oligonucleotides consisted of ten nucleotides that complemented sequences from either the T7 promotor or the M13 reverse sequencing primer. The 3′ domains of these oligonucleotides were 8-mer sequences predicted to anneal frequently to the protein-coding regions of mRNAs in a permiscuous fashion (Lopez-Nieto and Nigam, 1996). These oligonucleotides were then used in a sequential pairwise strategy that optimizes the amount of mRNA complexity that can be surveyed with limited numbers of primers and starting RNA. Care was taken to ensure that the two oligonucleotides used to produce any single fingerprint did not share sequence similarity in either their 5′ or 3′ domains. Because these oligonucleotides were constructed of short sequence domains that have specific functions within this experimental design, the oligonucleotides are permiscuous rather than truly arbitrary in nature.

Two RNA pools were fingerprinted. These two pools were each created by combining equal amounts of peripheral blood total RNA from five individuals. One pool was constructed by pooling RNA from five healthy individuals while the other pool was derived from five individuals with recurring metastatic prostate cancer. Using the pooled RNAs as templates, first strand cDNA synthesis was primed by annealing one of the permiscuous oligonucleotide primers to the pooled RNAs at low stringency. All fingerprinting studies were performed in duplicate using different initial concentrations of template RNA. The replicate fingerprints were initiated by using either 125 ng or 250 ng of RNA as template during first strand cDNA synthesis. Reaction conditions for first strand cDNA synthesis were 250 units of SuperScript II™ (GIBCO-BRL Gaithersburg, MD) in 1X supplier' s reaction buffer (25 mM Tris-HCl [pH=8.3], 37.5 mM KCl, 3.0 mM $MgCl_2$), 10 mM DTT, 400 μM each dNTP, and 2.0 μM permiscuous oligonucleotide in a 40 μl volume. The latter was incubated for 1 h at 37°C. Following first strand cDNA synthesis, the RNA was digested with RNase H and heat inactivated at 70°C as directed by the supplier.

One-tenth (4.0 μl) of the first strand cDNA reaction mixture was used in the fingerprinting PCR™ reaction. As many as ten different RNA fingerprints were generated from each first strand cDNA reaction. To the first strand cDNA, 36 μl of a PCR™ mix solution was added. The latter contained 50 mM Tris-Cl (pH=8.3), 50 mM KCl, 200 μM each dNTP, 1.0/μCi of α^{33} P-dCTP, 2.0 μM second permiscuous oligonucleotide and 1.0 unit of recombinant Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). Note that the concentration of the first oligonucleotide is now slightly less that 200 nM. PCR™ fingerprinting was performed with one cycle of 94°C for 2:00 min, 48°C for 5:00 min then 72°C for 5:00 min. This was followed by 35 cycles of 94°C for 45 sec, 48°C for 1:30 min, and 72°C for 2:00 min. A final extension step of 72°C for 5:00 was performed. Next, 4.0 μl of the final PCR™ products were mixed with 6.0 μl of sequencing formamide dye solution and denatured by heating to 75°C for 5:00 min. Approximately 2.5 μl of the denatured PCR™ products in formamide dye was electrophoresed through a 6% polyacrylamide, 7M urea DNA sequencing gel. PCR™ products were visualized by autoradiography.

The two differentially appearing PCR™ amplified cDNA fragments identified in these studies that are the subjects of this report were termed UC331 and UC332. UC331 was

identified in a study in which the first permiscuous primer used in the reverse transcription reaction had the sequence 5′ ACGACTCACTATAAGCAGGA 3′ (SEQ ID NO:24). The second permiscuous primer that was used in the PCR™ fingerprinting reaction that identified UC331 was 5′ AACAGCTATGACCATCGTGG 3′ (SEQ ID NO:25). UC332 was identified in a study in which the first permiscuous primer used in the reverse transcription reaction had the sequence 5′ ACGACTCACTATGTGGAGAA 3′ (SEQ ID NO:26). The second permiscuous primer that was used in the PCR™ fingerprinting reaction that identified UC332 was 5′ AACAGCTATGACCCTGAGGA 3′ (SEQ ID NO:27). After autoradiography, bands that appeared differentially in fingerprinting reactions on the pooled total RNAs described above were cut out of the gels and reamplified by PCR™. The reamplified PCR™ products were directly sequenced using the Sequenase™ reagent system (Amersham Life Sciences, Inc., Arlington Heights, IL.).

The sequences of UC331 and UC332 were compared to those deposited in release 101 of GenBank (July 1997) using the Lasergene™ software package (DNAstar, Inc., Madison, WI). The DNA sequence of these cDNA fragments, when compared to the GenBank database, revealed that the mRNAs, from which these cDNA fragments were derived, were previously uncharacterized. Neither UC331 nor UC332 are genes whose products have been previously characterized as being significant in any physiological pathway, both UC331 and UC332 match sequences on the GenBank data base.

In the case of UC331, these matches are confined to ESTs. UC331 was identical within the limits of sequencing accuracy to several human EST sequences. The human EST sequences with high similarity to UC331 could be assembled into a virtual contig that predicts the sequence of a larger mRNA. The ends of the UC331 contig were then used to requery the EST data base whereby more ESTs were identified that extended the contig. This process was continued until the UC331 contig predicted a mRNA with an ORF and a poly-A tail. A description of the human ESTs that were used to construct the UC331 contig are provided in Table 9. The sequence of the UC331 contig and the ORF was identified at its 5′ end. A significant feature of this contig is that the ORF extends all the way to its 5′ end. This indicates that the UC331 mRNA extends further 5′ than is indicated by the contig constructed from the EST database.

TABLE 9
UC331 EST Distribution
Human

GB Accession Number	Tissue	Library
AA403120	Total Fetus	Soares
AA401845	Total Fetus	Soares
AA121473	Pregnant Uterus	Soares
AA121262	Pregnant Uterus	Soares
R22145[i]	Placenta	Soares
R22146[i]	Placenta	Soares
R30954[i]	Placenta	Soares
R31006[i]	Placenta	Soares
R32887[h]	Placenta	Soares
R31390[h]	Placenta	Soares
R67806[g]	Placenta	Soares
R67807[g]	Placenta	Soares
AA385620	Thyroid	TIGR
W37985	Parathyroid Tumor	Soares
W37986	Parathyroid Tumor	Soares
AA380401	Cell line (Supt)	TIGR
AA182471	Cell line (HeLa)	Stratagene (IMAGE)
AA181530	Cell line (HeLa)	Stratagene (IMAGE)
W31231	Senescent Fibroblasts	Soares
N22701	Normal Melanocyte	Soares
N31175	Normal Melanocyte	Soares
N34446	Normal Melanocyte	Soares
N34538	Normal Melanocyte	Soares
N36424	Normal Melanocyte	Soares
N36521	Normal Melanocyte	Soares
N42854	Normal Melanocyte	Soares
N44299	Normal Melanocyte	Soares

GB Accession Number	Tissue	Library
W56398	Normal Melanocyte	Soares
N66813	Normal Melanocyte	Soares
AA379996	Skin Tumor	TIGR
AA370040	Prostate Gland	TIGR
AA369851	Prostate Gland	TIGR
H08822[k]	Brain (Whole infant)	Soares
H08905[k]	Brain (Whole infant)	Soares
H19533	Brain (Whole Adult)	Soares
H21379[f]	Brain (Whole Adult)	Soares
H21421[f]	Brain (Whole Adult)	Soares
H24360[e]	Brain (Whole Adult)	Soares
H25176[e]	Brain (Whole Adult)	Soares
H38689	Brain (Whole Adult)	Soares
H38791	Brain (Whole Adult)	Soares
H39147[d]	Brain (Whole Adult)	Soares
H39148[d]	Brain (Whole Adult)	Soares
H45092[c]	Brain (Whole Adult)	Soares
H45054[c]	Brain (Whole Adult)	Soares
H49928	Brain (Whole Adult)	Soares
H50463	Brain (Whole Adult)	Soares
H51403[a]	Brain (Whole Adult)	Soares
H51444[a]	Brain (Whole Adult)	Soares
H52811[b]	Brain (Whole Adult)	Soares
H52774[b]	Brain (Whole Adult)	Soares
R85542	Brain (Whole Adult)	Soares
R84652	Brain (Whole Adult)	Soares
AA324855	Brain (Cerebellum)	TIGR
AA317211	Retina	TIGR
AA371911	Pituitary Gland	TIGR
AA302113	Endothelial Cells, Aorta	TIGR
AA247643	Fetal Heart	U. Toronto
W60049	Fetal Heart	Soares
W61359	Fetal Heart	Soares

GB Accession Number	Tissue	Library
AA243511	B-Cells	Soares
AA234769	Pooled; fetal heart, melanocytes, pregnant uterus	Soares
AA 158239	Pancreas	Stratagene (IMAGE)
AA 150565	Pancreas	Stratagene (IMAGE)
AA 160836	Pancreas	Stratagene (IMAGE)
H73822	Fetal Liver Spleen	Soares
N58180	Fetal Liver Spleen	Soares
W04414	Fetal Liver Spleen	Soares
N94254	Fetal Liver Spleen	Soares
N75996	Fetal Liver Spleen	Soares
N69644	Fetal Liver Spleen	Soares
T83329	Fetal Liver Spleen	Soares
T72755	Fetal Liver Spleen	Soares
T53976	Pooled Fetal Spleens	Soares
N76701	Multiple Sclerosis	Soares
N90814	Multiple Sclerosis	Soares
N63292	Multiple Sclerosis	Soares
N59233	Multiple Sclerosis	Soares
N53207	Multiple Sclerosis	Soares
N51545	Multiple Sclerosis	Soares
F22624	Skeletal Muscle.	CRIBI (Italy)

Note:Paired superscripts indicate opposite ends of the same cDNA clone.

When the human UC331 contig was used to query the GenBank database many mouse EST sequences were identified with significant similarity. This was especially true in the region spanning the putative ORF. The identified mouse ESTs were found to have areas of overlap and similarity with each other that permitted them to be assembled into a mouse UC331 virtual

contig in a process that was identical to that used to create the human contig. The mouse UC331 virtual contig was also observed to have an ORF at its 5' end and a poly-A tail at its 3' end. A description of the mouse ESTs that were used to construct this contig are provided in Table 10.

TABLE 10
Mouse

GB Accession Number	Tissue	Library	Clone #
AA027487	Placenta	Soares	459407 (5')
AA023708	Placenta	Soares	456984 (5')
AA023154	Placenta	Soares	456027 (5')
AA024303	Placenta	Soares	458313 (5')
W35948	Total Fetus	Soares	350258 (5')
W 11581	Total Fetus	Soares	318665 (5')
W36820	Total Fetus	Soares	336707 (5')
AA002492	Mouse Embryo	Soares	426498 (5')
AA097370	Mouse Embryo	Soares	493073 (5')
AA014313	Mouse Embryo	Soares	468491 (5')
AA450512	Beddington embryonic region	IMAGE	865186 (5')
AA408179[L]	Embryo Ectoplacental Cone	Ko	C0025F09 (3')
AA408261[L]	Embryo Ectoplacental Cone	Ko	C0025F09 (5')
AA117174	T-cells	Stratagene	558134 (5')
AA119346	Thymus	Soares	573567 (5')
AA183195	Lymph Node	Soares	636222 (5')
AA122933	Kidney	Barstead	579415 (5')
AA423613	Mammary Gland	Soares	832219 (5')

Note:Paired superscripts indicate opposite ends of the same cDNA clone.

When the MegAlign™ program of the Lasergene™ DNA analysis software package (DNAstar, Inc.) was used to compare the mouse and human UC331 contigs, the two contigs were predicted to represent mRNA species that were highly similar and nearly collinear throughout their lengths. This similarity was most striking in the region comprising the putative ORFs. Within the ORFs the mouse and human contigs, the DNA sequences are 89% identical. In the

predicted 3′ untranslated regions of the two contigs, the DNA sequence similarity falls to 73% with several small deletions and insertions. This higher degree of sequence similarity in the putative ORFs as compared to the proposed 3′ untranslated region is interpreted as evidence that the ORFs encode proteins on which natural selection constrains amino acid sequence divergence. Like the human UC331 contig, the mouse contig also encodes a putative ORF that extends all the way to its 5′ end. This provides additional support for the contention that the UC331 mRNA contains more sequences at its 5′ end than are represented by the EST based contigs presented here.

The ORFs of the mouse and human UC331 contigs were conceptually translated and the amino acid sequences were compared. The amino acid sequence of the human UC331 ORF was used to query the Swiss, PIR and Translation release 101 using the Lasergene™ software package. For the 157 amino acids for which this comparison is possible, the mouse and human sequences are collinear and identical at 151 positions (96%) with five of the six differences being conservative substitutions. This putative protein domain is highly acidic with 26 acidic and 17 basic amino acids. There were also 48 hydrophobic and 41 polar amino acids predicted. When either the predicted mouse or human UC331 amino acid sequences was compared to amino acid sequences in the public protein sequence data bases, no significant matches were found to any previously characterized vertebrate proteins. However, a significant match was observed to a putative protein, termed ZK353.1 (PIR Accession number S44654), encoded in the genome of the nematode, *Caenorhabditis elegans*. The mammalian amino acid sequence is similar and collinear with the C-terminal 157 amino acids of the putative *C. elegans* protein. Like the mammalian UC331 amino acid sequences, the C-terminal 157 amino acid sequence of the ZK353.1 is also highly acidic with 31 acidic and only 20 basic amino acids. Over the 203 amino acids for which a comparison can be made the ZK353.1 amino acid sequence is identical to the human or mouse sequence at 84 (41%) positions with many of the differences representing conservative substitutions.

The putative *C. elegans* protein, ZK353.1, has no currently known function. Its existence is predicted from the *C. elegans* genome sequencing effort (Sulston *et al.*, 1992). The polypeptide sequence for ZK353.1 is a conceptual translation of an area on the *C. elegans* chromosome III (GB accession number CELZK353). The predicted sequence for ZK353.1 is

548 amino acids long and includes an additional 371 amino acids that are N-terminal of the domain with similarity to the predicted amino acid sequence of UC331. If UC331 is the mammalian homolog of ZK353.1 and if UC331 is collinear with the *C. elegans* protein over its entire length, it could be expected that the ORF of UC331 would extend roughly an additional 1100 nucleotides 5′ of the sequence in SEQ ID NO:29. While it is likely that the UC331 ORF extends further 5′ than is accounted for in the virtual mouse and human UC331 contigs, Northern blot data from human poly-A plus RNA discussed below indicates that the human UC331 mRNA extends only about 350 nucleotides further 5′. This may indicate an error in interpreting the possible pattern of 'mRNA processing from the *C. elegans* sequence or indicate simply that the mammalian and nematode mRNAs and encoded proteins are significantly different from each other at their 5′ and N-terminal ends respectively.

To confirm that the human UC331 virtual contig accurately represented the sequence of an authentic mRNA, oligonucleotides were designed to direct the PCR™ amplification of large cDNA fragments predicted to be continuous from the virtual contig but which contain significantly more sequence than can be found in any single EST.

UC332 did not match any EST sequences but was identical to a portion of a previously sequenced full length cDNA with a GenBank accession number of D87451.

RELATIVE QUANTITATIVE RT-PCR™

Frequently, mRNAs identified by RNA fingerprinting or differential display as being differentially regulated turn out not to be so when examined by independent means. It is, therefore, critical that the differential expression of all mRNAs identified by RNA fingerprinting be confirmed as such by an independent methodology. To independently confirm the differential expression of UC331 in the peripheral blood of patients with recurrent metastatic cancer compared to the peripheral blood of healthy volunteers, two different formats for a relative quantitative RT-PCR™ were performed. The first format of this assay examined normalized pools of cDNA constructed by combining equal amounts of cDNA from various individuals representing similar physiologic states. In this study, a cDNA pool representing 8 healthy volunteers was compared to a pool representing 10 individuals with recurrent metastatic prostate cancer. A third pool representing 10 individuals with recurrent metastatic breast cancer was also

examined. The inclusion of the breast cancer patient samples in this study was made to determine if the mRNAs examined were being differentially regulated in the immune system in a response that was specific for prostate cancer or if the response was more general to metastatic cancer in general. Using these pools of cDNA as templates, triplicate PCR™ was performed. Each of the three replicates were terminated at a different cycle number of PCR™. This format of relative quantitative RT-PCR™ insures that the results taken for relative quantitation represent the PCRs™ when they are in the log linear portions of their amplification curves where such quantitation is most accurate.

Approximately 1.5-5.0 μg of DNA-free total RNA from the peripheral blood of healthy volunteers or patients with either metastatic prostate or breast cancer were converted into first strand cDNA using the *SuperScript™ Preamplification System* for First Strand cDNA Synthesis (GIBCO-BRL, Cat# 18089-011) following the directions provided by the supplier. These cDNAs were then normalized to contain equal concentrations of amplifiable cDNA by PCR™ amplification of β-actin cDNA using the primers 5′ GGAGCTGCCTGACGGCCAGGTCATC 3′ (SEQ ID NO:28) and 5′ GAAGCATTTGCGGTGGACGATGGAG 3′ (SEQ ID NO:9). A typical PCR™ program would be 94°C for 1:15 min, followed by 22 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1:15 min. This was followed by final extension of 72°C for 5:00 min. PCR™ products were visualized by gel electrophoresis through 1.5% agarose TAE gels stained with ethidium bromide. Images of the gels were captured, digitized and analyzed using the IS-1000 Digital Imaging System (Alpha Innotech Corp.). The concentrations of the cDNAs were adjusted by adding various amounts of water to create cDNA stocks that contained equal concentrations of amplifiable β-actin cDNA. Typically, the cDNA derived from the reverse transcription of 5.0 μg of RNA resulted in enough normalized cDNA to perform 50-200 RT-PCR™ reactions.

Equal amounts of the normalized cDNA stock from individuals having the same disease state were pooled. Pools of cDNAs from healthy volunteers, patients with metastatic prostate cancer and metastatic breast cancer were produced. These pools were then examined by PCR™ for β-actin to determine that they contained equal amounts of amplifiable cDNA.

To demonstrate that all observations were made in the log-linear phase of the PCR™ amplification curve, a series of PCR™ reactions using different cycle number were performed on

each cDNA pool for each gene (primer pair) examined. Display of the PCR™ products on electrophoretic gels and analysis with the IS 1000 Digital Imaging System illustrates that the mass of the PCR™ products is increased exponentially with increasing cycle number, confirming that the observed results are in the log-linear portion of the PCR™ amplification curve.

Relative quantitative RT-PCR™ showing near equal amounts of amplifiable β-actin cDNA in three pools cDNA. Pools of normalized cDNAs were constructed from peripheral blood RNAs from eight healthy volunteers, ten individuals with recurrent metastatic prostate cancer, or ten individuals with recurrent metastatic breast cancer. Three separate PCR™ reactions were performed on each pool of cDNA. PCR™ was terminated at differing cycle numbers (cycle 22, cycle 24, and cycle 26), and the products were visualized by electrophoreses and ethidiumn bromide staining. Images were captured and quantitated using a digital image analysis system. At all three cycle numbers examined, there are relatively similar band intensities representing the three cDNA pools and increasing band intensity with increasing cycle number, verifying that the observations are being made in the log linear range of the amplification curves. Similar band intensities indicate similar relative concentrations of β-actin mRNA in the RNAs from individuals from which these cDNA pools were constructed.

The oligonucleotides used in the relative quantitative RT-PCR™ studies that independently confirmed the differential expression of UC331 were designed from the sequence in the human UC331 virtual contig. These UC331 specific oligonucleotides had the sequences of 5′ CTGGCCTACGGAAGATACGACAC 3′ (SEQ ID NO:31) and 5′ ACAATCCGGAGGCATCAGAAACT 3′ (SEQ ID NO:32). These oligonucleotides direct the amplification of a 277 nucleotide long PCR™ product that is specific for UC331. The oligonucleotides used in the relative quantitative RT-PCR™ studies that independently confirmed the differential expression of UC332 were designed using the sequences of the cDNA with the GenBank accession number D87451. These UC332 specific oligonucleotides had the sequences 5′ AGCCCCGGCCTCCTCGTCCTC 3′ (SEQ ID NO:33) and 5′ GGCGGCGGCAGCGGTTCTC 3′ (SEQ ID NO:34). These oligonucleotides direct the amplification of a 140 nucleotide long PCR™ product that is specific for UC332.

The results for relative levels of β-actin expression contrasts sharply with those observed when oligonucleotide primers specific for UC331 were used to direct PCR™ amplification (FIG.

7). At 25 cycles of PCR™, clear bands are visible in the lanes representing the pools of cDNA from peripheral blood of patients with either metastatic breast or prostate cancer. In the lane representing the peripheral blood of healthy volunteers, only a very faint band is present. At 28 cycles of PCR™, the band intensities representing all three pools are brighter than they were at 25 cycles, but the relative increase in intensity of the bands representing the metastatic cancer patient pools compared to the healthy volunteers remains the same as was observed at 25 cycles of PCR™. This indicates that these observations are being made in the log linear range of the PCR™ amplification curves. At 31 cycles of PCR™, there is still an increase in the intensity of the bands representing the pools of metastatic cancer patients compared to the pool representing the healthy volunteers, but a quantitative analysis of these bands indicates that the PCRs™ have left the log linear range of their amplification curves. Quantitation of the data for 25 and 28 cycles of PCR™ independently confirms that UC331 mRNA is differentially regulated and is roughly seven fold more abundant in the peripheral blood leukocytes of the average patient with either recurrent metastatic prostate cancer or breast cancer than in the peripheral blood leukocytes of healthy volunteers.

The second format of relative quantitative RT-PCR™ used to examine the differential expression of UC331 examined the relative abundance of UC331 mRNA in the peripheral blood of healthy individuals or individuals with recurrent metastatic cancer. The individuals examined in this study were the same as those whose cDNAs were combined to construct the pools examined as described above. Using the information obtained from the pooled cDNA study to predict at what PCR™ cycle numbers relative quantitative RT-PCR™ would be most informative, these individuals were examined for the relative abundance of β-actin and UC331 mRNAs present in their peripheral blood leukocytes. PCR™ was for 22 cycles. All individuals examined contain roughly equal amounts of amplifiable β-actin cDNA. Some of the differences in β-actin band intensity observed in this study are probably due to the internal variation inherent of this study. Results from studies designed to quantitate this internal variation indicate that identical replicates of a β-actin PCR™ can be expected to vary in the intensity of product bands with a standard deviation of ±15%.

Relative quantitative RT-PCR™ of UC331 cDNA was conducted using reverse transcribed from RNA isolated from the peripheral blood of eight healthy volunteers (group N),

ten individuals with recurrent methstatic prostate cancer (group P), or ten individuals with recurrent metastatic breast cancer (group B). PCR™ was for 30 cycles. As was seen in the study using the pooled cDNAs, the results of the relative quantitative RT-PCR™ for UC331 using cDNA from individuals contrasts sharply with that observed for β-actin. The intensity of the band representing the abundance of the UC331 mRNA in peripheral blood leukocytes was greater for all of the patients with either metastatic prostate or breast cancer as compared to the intensity of the UC331 band representing the mRNA level in the peripheral blood leukocytes of healthy volunteers. Therefore, the elevated UC331 mRNA levels indicated by the relative quantitative RT-PCR™ results using the pooled cDNA templates was caused by an elevated mRNA level in all individuals comprising the pools and not from a subset of individuals with very high elevations in UC331 mRNA levels. This study is a second independent confirmation of the differential expression of the UC331 mRNA.

As is indicated by the wide distribution of tissues from which the ESTs used to assemble the UC331 contigs (Table 9), UC331 is widely expressed in many tissue and cell types. However, because most of ESTs comprising UC331 are from normalized libraries, little information can be gained from this data on the relative abundance of the UC331 mRNA in different tissues. Also, while the extension of the ORFs of the mouse and human UC331 contigs all the way to their 5′ ends and the similarity of mammalian UC331 mRNAs to a much larger putative *C. elegans* mRNA both predict that the mammalian UC331 mRNA extends even further 5′, the exact size of the UC331 mRNA was unknown. To address all of these issues, a Northern blot of poly-A plus RNA from eight different human tissues was probed with the 850 nucleotide long RT-PCR™ product described above labeled with ^{32}P. Approximately 2.0 μg of poly-A plus RNA from spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes were loaded in each lane. UC331 mRNA is expressed in all eight human tissue and cell types. Size standards indicate a message size of approximately 1.75 kb. Interestingly, UC331 is least abundant in peripheral blood leukocytes but is highly expressed in the thymus, demonstrating a difference in expression between cells of different developmental stages in the immune system. UC331 is most abundantly expressed in the testes. The UC331 mRNA is about 1.75 kb which indicated that the mRNA only extends about 350 nucleotides further 5′ than is accounted for by the virtual contig shown in SEQ ID NO:29. The translation product of the

virtual contig is shown in SEQ ID NO:30. Clearly, the putative *C. elegans* mRNA extends much more 5′ than do the mammalian mRNA species.

The other gene identified as being differentially regulated in this RNA fingerprinting study was UC332. UC332 was analyzed in much the same way as UC331 was. When the sequence of the cDNA fragment from the RNA fingerprinting gel representing UC332 was used to query GenBank, no ESTs were identified. The sequence of the UC332 cDNA fragment did, however, identify a sequence of a full length cDNA, KA000262 (GB:accession number D87451). The sequence of KA000262, (hereafter referred to interchangeably with the name, UC332) was determined as part of a project to examine previously unidentified mRNAs expressed in the bone marrow myeloblast cell line, KG-1 (Nagase *et al.*, 1996). This mRNA contains an ORF encoding a putative protein with 761 amino acid sequence. Perhaps the most striking feature of this polypeptide sequence is the appearance of a C3HC4 RING zinc finger or RING finger motif (Freemont, 1993) located between amino acids 175 and 216. The RING finger domain binds two zinc ions in a conserved structure that has been resolved (Barlow *et al.*, 1994). RING finger domains have been identified in dozens of proteins derived from eukaryotes as diverse as yeasts, flies, birds, nematodes and humans. In most of these cases, the RING finger containing proteins have been shown to be essential for some important biological process although the these processes vary considerably one from another. Among these mammalian encoded RING finger proteins are several genes implicated in the ontogeny of cancer including the *ret* viral oncogene (Takahashi *et al.*, 1988) and bmi-1, a gene whose product collaborates with myc induced transformation (Haupt *et al.*, 1991). The BRCA-1 tumor suppressor gene involved in hereditary breast and ovarian cancer susceptibility contains a RING finger domain (Miki *et al.*, 1994), and MAT-1, a novel 36 kDa RING finger protein, is required for the assembly of enzymatically active CDK7- cyclin H complexes (Tassan *et al.*, 1995). A comparison of the RING finger domains of UC332 and various representative members of this group, including BRAC1, rpt-1, Traf5, HT2A, MAT1, rfp, bmi-1, CRZF, and neu, indicates the RING finger domain of UC332 is slightly more similar to those found in the tumor suppressor gene, BRCA1, and the T cell repressor of transcription protein, rpt-1. However, BRCA1 and rpt-1 are more similar to each other than they are to UC332.

Proteins with RING finger motifs exhibit heterogeneity in their subcelluar localizations. Some, that are important regulators of differential gene regulation, localize to the cell nucleus. When the amino acid sequence of UC332 was scanned for evidence of subcellular localization, two domains were identified that contained sequences for putative nuclear localization signals (NLS). NLS are highly basic stretches of six are more amino acids of which at least four are basic that tend to be flanked by acidic amino acids and/or prolines (Boulikas, 1994). Both of the putative NLS in UC332 longer and more basic than the minimum requirements for the consensus NLS motif. The first of these putative NLS motifs occurs between amino acid 548 and 567. Within this domain, 13 of 19 amino acids are basic. In fact, this domain could be viewed as two NLS in tandem separated by two glutamic acid residues. If divided this way, the first NLS domain would have 8 of eleven positions as basic amino acids while the second motif would have 5 of 6 amino acids being basic. The second NLS motif in UC332 is located near the C-terminal end between positions 739 and 750 in the amino acid sequence. This domain has 8 of 12 amino acids as basic residues with a core of 5 consecutive lysines and arginines. The presence of these putative NLS in the amino acid sequence of UC332 suggest the possibility that UC332 plays an important role in regulating the expression of other genes. Finally, the amino acid sequence of UC332 lacks a signal sequence for cellular export or an obvious hydrophobic transmembrane domains.

To independently verify that UC332 mRNA is more abundant in the peripheral blood leukocytes of patients with recurrent metastatic cancer as compared to the peripheral blood leukocytes of healthy volunteers, relative quantitative RT-PCR™ was performed using the same cDNAs and formats as were used to investigate the differential regulation of UC331. A relative quantitative RT-PCR™ study using UC332 specific oligonucleotide primers and cDNA pools as templates was conducted. At 25 and 28 cycles of PCR™, the amplified DNA band representing the relative abundance of the UC332 mRNA is stained more intensely for those reactions that used cDNA template pools constructed from the peripheral blood leukocyte RNA isolated from metastatic prostate and breast cancer patients as compared to a similar pool constructed from RNA from healthy volunteers. Quantitation of this image using the IS-1000 Digital Imaging System (Alpha Innotech, Inc.) indicates that UC332 mRNA is roughly 5 times more abundant in

the peripheral blood leukocytes of metastatic cancer patients compared to healthy volunteers. At 31 cycles of PCR™, the reactions have left the log linear range of their amplification curves.

In a second relative quantitative RT-PCR™ study using UC332 specific oligonucleotide primers, peripheral blood leukocyte cDNA from the individuals that comprised the pools from the peripheral blood of eight healthy volunteers, ten individuals with recurrent metastatic prostate cancer, or ten individuals with recurrent metastatic breast cancer were examined separately. PCR™ was for 26 cycles. The results of this study are similar to those obtained when the pooled cDNAs were used as PCR™ templates. All of the cancer patients had higher levels of UC332 mRNA in their peripheral blood leukocytes than did any of the healthy volunteers.

In this study, the inventors showed that UC332, encoding a RING finger protein, is up regulated in the peripheral blood leukocytes of patients with either recurrent metastatic breast or prostate cancer. From the literature, RING finger proteins have been shown to participate in the regulation of several important lymphocytic processes (Patarca *et al.*, 1988; Fridell *et al.*, 1995; Takeuchi *et al.*, 1996; van Arsdale *et al.*, 1997; Nakano *et al.*, 1996). The observed differential regulation of the RING protein encoding mRNA, UC332, in the immune response of patients with metastatic breast or prostate cancer strongly suggests that UC332 participates in regulating this immune response.

All of the compositions and methods disclosed and claimed herein may be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of preferred embodiments, it is apparent that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention.

More specifically, it is apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the disclosure as defined by the appended claims.

UC325-1 is derived from the IL-8 gene (Genebank Accession #M28130). UC325-1 and UC325-2, an alternatively spliced form that includes the third intron of the IL-8 primary transcript,

are transcribed from the IL-8 gene. Our definition of IL-8 gene products means all mRNAs transcribed from the IL-8 gene, the polypeptides encoded by those mRNAs and their post-translationally processed protein products.

Those practiced in the art will realize that there exists naturally occurring genetic variation between individuals. As a result, some individuals may synthesize IL-8 gene products that differ from those described by the sequences entailed in the Genebank number listed above. We include in our definition of IL-8, those products encoded by IL-8 genes that vary in sequence from those described above. Those practiced in the art will realize that modest variations in DNA sequence will not significantly obscure the identity of a gene product as being derived from the IL-8 gene.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Abbondanzo *et al.*, *Breast Cancer Res. Treat.*, 16: 182(#151), 1990.

Adams *et al.*, 1995.

Adams, Kelley, Gocayne, Dubnick, Polymeropoulos, Xiao, Merril, Wu, Olde, Moreno, Kerlavage, McCombie, Venter, "Complementary DNA sequencing: Expressed sequence tags and he human genome," *Science*, 252:1651-1656, 1991.

Adams, Kerlavage, Fleischmann, Fuldner, *et al.*, "Initial assessment of the human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence," *Nature 377: Supplement: The Genome Directory*, pages 3-174.

Alcaraz *et al.*, *Cancer Res.*, 55:3998-4002, 1994.

Allred *et al.*, *Breast Cancer Res. Treat.*, 16:182(#149), 1990.

An *et al.*, *Proc. Amer. Assn. Canc. Res.*, 36:82, 1995.

Andrawis *et al.*, *Proc. Am. Urol. Assn.* 155(5): 512A, 1996.

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, Cold Spring Harbor, New York, 1988.

Barinaga, *Science*, 271: 1233, 1996.

Barlow, Luisi, Milner, Elliott, Everett, "Structure of the C3HC4 Domain by H-nuclear Magnetic Resonance Spectroscopy," *J. Mol. Biol.*, 237:201-211, 1994.

Barry *et al.*, *Urology*, 46:2-14, 1995.

Beck, Kattan and Miles, *J. Urol.*, 152:1894-99, 1994.

Bellus, *J. Macromol. Sci. Pure Appl. Chem*, A31(1): 1355-1376, 1994.

Bittner *et al.*, *Methods in Enzymol*, 153: 516-544, 1987.

Bookstein *et al.*, *Proc. Nat'l Acad. Sci. USA*, 87:7762-7767, 1990b.

Bookstein *et al.*, *Science*, 247:712-715, 1990a.

Boring *et al.*, *CA-Cancer J. Pract.*, 43:7-26, 1993.

Boulikas, "Putative nuclear localization signals (NLS) in protein transcription factors," *J. Cell. Biol.*, 55:32-58, 1994.

Bova *et al.*, *Cancer Res.*, 53:3869-3873, 1993.

Brawn *et al.*, *The Prostate*, 28: 295-299, 1996.

Breiman *et al.*, "Classification and Regression Trees," Pacific Grove: Wadsworth, 1984.

Brown *et al.*, *Breast Cancer Res. Treat.*, 16:192(#191), 1990.

Campbell, *In: Monoclonal Antibody Technology, Laboratory Techniques in Biochemistry and Molecular Biology*, Burden and Von Knippenberg, Eds., Vol. 13:75-83, Elsevier, Amsterdam, 1984.

"Cancer facts and figures-1997," American Cancer Society Surveillance Research, Pp. 4-5, 1997.

Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, *76*: 425, 1977 .

Carter and Coffey, *In: Prostate Cancer: The Second Tokyo Symposium*, J.P. Karr and H. Yamanak (eds.), pp. 19-27, New York: Elsevier, 1989.

Carter and Coffey, *Prostate*, 16:39-48, 1990.

Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 93: 749-753, 1996.

Carter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 87:8751-8755, 1990.

Catalona *et al.*, *J. Urol.*, 151:1283-1290, 1994.

Catalona *et al.*, *J.A.M.A.*, 274:1214, 1995.

Chen *et al.*, *Clin. Chem.*, 41:273-282, 1995.

Chen *et al.*, *Proc. Am. Urol. Assn.*, 153:267A, 1995.

Chinault and Carbon, *Gene*, 5:111-126, 1979.

Chomczynski and Sacchi, *Anal. Biochem.*, 162: 156-159, 1987.

Christensson *et al.*, *J. Urol.*, 150:100-105, 1993.

Colberre-Garapin *et al.*, *J. Mol. Biol.*, 150: 1, 1981.

Damaj *et al.*, *FASEB J.* 10: 1426-1434, 1996.

Davey *et al.*, EPO No. 329 822.

Dhom, *J. Cancer Res. Clin. Oncol.*, 106:210-218, 1983.

di Celle *et al.*, *Blood*, 84: 220-228, 1994.

Diamond *et al.*, *J. Urol.*, 128: 729-734, 1982.

Donahue *et al.*, *J. Biol. Chem.*, 269:8604-8609, 1994.

Dong *et al.*, *Science*, 268: 884-886, 1995.

Dugan *et al.*, *J.A.M.A.*, 275(4):288-294, 1996.

Dumont *et al.*, *J. Immunol.*, 152:992-1003, 1994.

Everett, O'Hara, O'Rourke, Barlow, Orr, "Point mutations in the herpes simplex virus type 1 Vmw1 10 RING finger helix affect activation of gene expression, viral growth, and interaction with PML-containing nuclear structures," *J. Virology*, 69:7339-7344, 1995.

Freemont, "The RING finger: A novel protein sequence motif related to the zinc finger," *Ann. N.Y. Acad. Sci.*, 684:174-192, 1993.

Freifelder, *Physical Biochemistry Applications to Biochemistry and Molecular Biology*, 2nd ed., Wm. Freeman and Co., New York, NY, 1982.

Fridell, Harding, Bogerd, Cullen, "Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins," *Virology*, 209:347-357.1995.

Frohlich *et al.*, *Molec. Cell. Biol.*, 10:3216-3223, 1990.

Frohman, *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*. Academic Press, N.Y., 1990.

Fujichima *et al.*, *Cytometry*, 24:382-389, 1986.

Garnick, *Scientific Amer.* 270: 72-81, 1994.

Gefter *et al.*, *Somatic Cell Genet.*, 3: 231-236, 1977.

Gingeras *et al.*, PCT Application WO 88/10315.

Goding, *In: Monoclonal Antibodies: Principles and Practice*, 2d ed., Orlando, Fla., Academic Press, pp. 60-61, 65-66, 71-74, 1986.

Haupt, Alexander, Barri, Klinken, Adams, "Novel zinc finger gene implicated as *myc* collaborator by retrovirally accelerated lymphomagenesis in *Ep.-myc* transgenic mice," *Cell*, 65:753-763, 1991.

Hedges *et al.*, *Cytokine* 8:686-697, 1996.

Hess *et al.*, *J. Adv. Enzyme Reg.*, 7:149, 1968.

Heston, personal communication, CapCURE meeting, Lake Tahoe, CA, 1996.

Hitzeman *et al.*, *J. Biol. Chem.*, 255:2073, 1980.

Holland *et al.*, *Biochemistry*, 17:4900, 1978.

Holmes *et al.*, *Prostate*, 27:25-29, 1996.

Horoszewicz, Kawinski and Murphy, *Anticancer Res.*, 7:927-936, 1987.

Hosmer and Lemeshow, *In: Applied Logistic Regression*, New York, John Wiley & Sons, 1989.

Huang *et al.*, *Prostate*, 23: 201-212, 1993.

Ikei *et al.*, *Cytokine* 4: 581-584, 1992.

IMAGE ref.

Innis *et al.*, *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990.

Inouye *et al.*, *Nucleic Acids Res.*, 13:3101-3109, 1985.

Isaacs *et al.*, *Cancer Res.*, 51:4716-4720, 1991.

Isaacs *et al.*, *Seminars in Oncology*, 21:1-18, 1994.

Israeli *et al.*, *Cancer Research*, 54:1807-1811, 1994.

Issacs Reference

Johnson *et al.*, *In: BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York, 1993.

Jones, *Genetics*, 85:12, 1977.

Kingsman *et al.*, Gene, 7: 141, 1979.

Koch *et al.*, *Science*, 258:1798-1801, 1992.

Kohler and Milstein, *Eur. J. Immunol.*, 6: 511-519, 1976.

Kohler and Milstein, *Nature*, 256:495-497, 1975.

Kurdowska *et al.*, *J. Immunol.*, 154:1930-1940, 1997.

Kwoh *et al.*, *Proc. Nat. Acad. Sci. USA*, 86: 1173, 1989.

Liang and Pardee, "Differential display of eukaryotic messenger RNA by means of polymerase chain reaction," *Science*, 257:967-971.1992.

Liang and Pardee, *Science*, 257: 967-971, 1992.

Liang and Pardee, U.S. Patent 5,262,311, 1993.

Liang *et al.*, *Cancer Res.*, 52:6966-6968, 1992.

Lifton, *Science*, 272: 676, 1996.

Lilja *et al.*, *Clin. Chem.*, 37:1618-1625, 1991.

Lopez-Nieto and Nigam, "Selective amplification of protein-coding regions of large sets of genes using statistically designed primer sets," *Nature Biotechnology*, 14:857-861, 1996.

Lowy *et al.*, *Cell*, 22:817, 1980.

Macoska *et al.*, *Cancer Res.*, 54:3824-3830, 1994.

Marley *et al.*, *Urology*, 48(6A):16-22, 1996.

McClelland *et al.*, *Nucl. Acids Res.* 22: 4419-4431, 1994.

McCormack *et al.*, *Urology*, 45:729-744, 1995.

Miki *et al.*, *Science*, 266: 66-71, 1994.

Miki, Swensen, Shatmck-Eiden, Futreal, Harshman, Tavigian, Liu, Cochran, Bennett, Ding, Bell, Rosenthai, Hussey, Tran, McClure, Frye, Hattier, Phelps, Haugen-Strano, Katcher, Yakumo, Gholami, Shaffer, Stone, Bayer, Wray, Bogden, Dayananth, Ward, Tonin, Narod, Bristow, Norris, Helvering, Morrison, Rosteck, Lie, Barrett, Lewis, Neuhauses, Cannon-Albright, Goldgar, Wiserman, Kamb, Skolnick, "A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1," *Science*, 266: 66-71, 1994.

Miller *et al.*, PCT Application WO 89/06700.

Mok *et al.*, *Gynecol. Oncol.*, 52: 247-252, 1994.

Montie, *Urology*, 44: 2-8, 1994.

Morahan *et al.*, *Science* 272: 1811, 1996.

Morise *et al.*, *Med. Dec. Making*, 16(2):133-142, 1996.

Morton *et al.*, *Cancer Res.*, 53:3585-3590, 1993.

Morton *et al.*, *In: CANCER MEDICINE* (3rd Ed.), Holland, J.F., Frei III, E., Bast Jr., C.C. (eds), Lea and Febiger, Philadelphia, PA, pp. 1793-1824, 1993.

Mulligan *et al.*, *Proc. Nat'l Acad. Sci. USA*, 78:2072, 1981.

Murphy *et al.*, *Cancer*, 78: 809-818, 1996.

Murphy *et al.*, *Prostate*, 26:164-168, 1995.

Nagase, Seki, Ishikawa, Ohira, Kawarabayasi, Ohara, Tanaka, Kotani, Miyajima, Nomura, "Prediction of the coding sequences of unidentified human genes. VI. The coding sequences of 80 new genes (KIAA0201-KIAA0280) deduced by analysis of cDNA clones from cell line KG-1 and brain," *DNA Res.*, 3(5):321-329, 1996.

Nakamura *et al.*, *In: Handbook of Experimental Immunology* (4th Ed.), Weir, E., Herzenberg, L.A., Blackwell, C., Herzenberg, L. (eds), Vol. 1, Chapter 27, Blackwell Scientific Publ., Oxford, 1987.

Nakano, Oshima, Chung, Williams-Abbott, Wares, Yagita, Okumura, "TRAF5, an activator of NF-κB an putative signal transducer for lymphotoxin-β receptor," *J. Biol. Chem.*, 271:14661-14664, 1996.

O'Hare *et al.*, *Proc. Nat'l Acad. Sci. USA*, 78:1527, 1981.

Oesterling *et al.*, *J. Urol.*, 154:1090-1095, 1995.

Oesterling, *Contemporary Urology*, 8:76-92, 1996.

Ohara *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:5673-5677, 1989.

Papsidero *et al.*, *Cancer Research*, 40:2428-2432, 1980.

Parker *et al.*, *CA Cancer J. Clin.*, 65:5-27, 1996.

Parker, Tong, Bolden, Wingo, "Cancer statistics," *CA Cancer J. Clin.*, 65:5-27, 1996.

Partin and Oesterling, (Eds.) *Urology*, 48(6A) supplement: 1-87, 1996.

Partin and Oesterling, *J. Urol.*, 152:1358-1368, 1994.

Partin *et al.*, *Cancer Res.*, 53:744-746, 1993.

Patarca, Scwartz, Singh, Kong, Murphy, Anderson, Sheng, Singh, Johnson, Guarnagia, Durfee, Blattner, Cantor, "rpt-1, an intracellular protein from helper/inducer T cells that regulates gene expression of interleukin 2 receptor and human immunodeficiency virus type 1," *Proc. Natl. Acad. Sci. USA*, 85:2733-2737, 1988.

Pearsons *et al.*, *J. Urol.*, 150:120-125, 1993.

Pettersson *et al.*, *Clin. Chem.*, 41:1480-88, 1995.

Piironen *et al.*, *Clin. Chem.* 42: 1034-1041, 1996.

Price, Chang, Smith, Bockheim, Laughon, "The Drosophila neutralized gene encodes a C3HC4 zinc finger," *EMBO J.*, 12:2411-2418, 1993.

Qiao *et al.*, *Biochem. Biophys. Res. Comm.*, 201:581-588, 1994.

Ralph *et al.* 1993.

Ribas de Pouplana and Fothergill-Gilmore, *Biochemistry*, 33:7047-7055, 1994.

Rieber, M. and Rieber, M. S., *Cell Growth Diff.*, 5:1339-1346, 1994.

Sager *et al.*, *FASEB J.*, 7:964-970, 1993.

Saito *et al.*, *Biochem. Biophys. Res. Commun.*, 200:378, 1994.

Sambrook *et al.*, (ed.), *MOLECULAR CLONING*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Schadendorf *et al.*, *J. Immunol.*, 151:2667-2675, 1993.

Scheibenbogen *et al.*, *Melanoma Research*, 5:179-181, 1995.

Schroder, J.M., Sticherling, M., Henneicke, H. H., Preissner, W. C., Christophers, E. *J. Immunol.* 144:2223-2232, 1990.

Scott *et al.*, *Molec. Cell. Biol.*, 13:2247-2257, 1993.

Sidransky *et al.*, *Science*, 252: 706-709, 1991.

Silver *et al.*, *Clin. Cancer Res.*, 3: 81-85, 1997.

Slamon *et al.*, *Science*, 224:256-262, 1984.

Slamon *et al.*, *Science*, 244: 707-712, 1989.

Smith, U.S. Patent No. 4,215,051

Stamey, *Urology*, 45(2):173-184, 1995.

Steinberg and Colla, *CART: A supplementary module for SYSTAT*, Evanston, IL, SYSTAT Inc., 1992.

Stenman *et al.*, *Cancer Research*, 51:222-226, 1991.

Stinchcomb *et al.*, *Nature*, 282:39, 1979.

Stone *et al.*, *Urology*, 44: 18-25, 1994.

Sulston, Du,. Thomas. Wilson, Hillier, Staden, Halloran, Green,. Thierry-Mieg, Qiu. Dear, Coulson, Craxton, Durbin, Berks, Metzstein, Hawkins, Ainscough, Waterston, "The *C. elegans* genome sequencing project: A beginning," *Nature*, 356:37-41, 1992.

Sun and Cohen, *Gene*, 137:127-132, 1993.

Szybalska *et al.*, *Proc. Nat'l Acad. Sci. USA*, 48: 2026, 1962.

Takahashi *et al.*, *Cancer Res.*, 54:3574-3579, 1994.

Takahashi, Inaguma, Hiai, Hirose, "Developmentally regulated expression of human "Finger"-containing gene encoded by the 5' half of the *ret* transforming gene," *Mol. Cell. Biol.*, 8:1853-1856, 1988.

Takeuchi, Roth, Goeddel, "Anatomy of TRAF2: Distinct domains for Nuclear Factor-κB activation and association with tumor necrosis factor signaling proteins," *J. Biol. Chem.*, 271:19935-19942, 1996.

Tan, Waterham, Veenhuis, Gregg, "The *Hansenula polymorpha* PER8 gene encodes a novel peroxisomal integral membrane protein involved in proliferation," *J. Cell Biol.*, 128:307-319, 1995.

Taparowsky *et al.*, *Nature*, 300: 762-764, 1982.

Tassan, Jaquenoud, Fry, Frutiger, Hughes, Nigg, "*In vitro* assembly of a functional human CDK7-cyclin H complex requires MAT1, a novel 36 kDa RING finger protein," *EMBO J.*, 14:4508-5617, 1995.

Thanos and Maniatis, "NF-κB: A lesson in family values," *Cell*, 80:529-532," 1995.

TIGR reference

Tranque, Crossin, Cirelli, Edelman, Mauro, "Identification and characterization of a RING zinc finger gene (C-RZF) expressed in chicken embryo cells," *Proc. Natl. Acad. Sci. USA*, 93:3105-3109, 1996.

Tschemper *et al.*, *Gene*, 10:157, 1980.

Tummuru, Sharma and Blaser, *Molec. Microbiol.* 18: 8670876, 1995.

Umbas *et al.*, *Cancer Res.*, 52:5104-5109, 1992.

VanArsdale, VanArsdale, Force, Walter, Mosialos, Kieff, Reed, Ware, "Lymphotoxic-β receptor signaling complex: Role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor κB," *Proc. Natl. Acad. Sci. USA*, 94:2460-2465, 1997.

Vinante *et al.*, *Leukemia*, 7:1552-1556, 1993.

Visakorpi *et al.*, *Am. J. Pathol.*, 145:1-7, 1994.

Vishwanatha *et al.*, *Carcinogenesis*, 14:2575-2579, 1993.

Vollmer, *Am. J. Clin. Pathol.*, 105:115-126, 1996.

Walker *et al.*, *Proc. Nat'l Acad. Sci. USA*, 89:392-396, 1992.

Walsh and Worthington, *The Prostate*, Chap. 3, Johns Hopkins University Press, 1995.

Wan, Sharp, Poirier, Wagaman, Chambers, Pyati, Hom, Galindo, Huvar, Peterson, Jackson, Erlander, "Cloning differentially expressed mRNAs," *Nature Biotechnology*, 14:1685-1691, 1996.

Watson *et al.*, *Cancer Res.*, 54:4598-4602, 1994.

Webb and Lin, *Invest. Urol.*, 17:401-404, 1980.

Welsh and McClelland, *Nucl. Acids Res.*, 18:7213-7218, 1990.

Welsh *et al.*, *Nucleic Acids Res.*, 20:4965-4970, 1992.

Welsh, Chada, Dalal, Chang, Ralph, McClelland, "Arbitrarily primed PCR™ fingerprinting of RNA," *Nucl. Acids Res.*, 20: 4965-70, 1992.

Whittemore, *Biometrika*, 82(1):57-67, 1995.

Wigler *et al.*, *Cell*, 11: 223, 1977.

Wigler *et al.*, *Proc. Nat'l Acad. Sci. USA*, 77:3567, 1980.

Wong *et al.*, *Int. J. Oncol.*, 3:13-17, 1993.

Wu *et al.*, *Genomics*, 4:560, 1989.

Yoon, Lee, Jongeward, Steinberg, "Similarity of sli-1, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene c-cbl," *Science*, 269:1102-1105, 1995.

Yoshimura, T., Robinson, E. A., Appella, E., Matsushima, K., Showalter, S.D., Skeel, A., and Leonard, E.J. *Molecular Immunology*. 26: 87-93, 1989.

Zhang *et al.*, *Clin. Chem.*, 41(11):1567-73, 1995.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: UROCOR, Inc.
(B) STREET: 800 Research Parkway
(C) CITY: Oklahoma City
(D) STATE: Oklahoma
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 73104

(ii) TITLE OF INVENTION: DIAGNOSIS OF DISEASE STATE USING mRNA PROFILES

(iii) NUMBER OF SEQUENCES: 34

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 253 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTGGCATGGC CCTGGAAAAC TGCGAAGTCT TCTCTCTGTG CAAACTTTCA CCTGGACTTT    120

TTATATGATT CTGGAAGTAT TCCAAGAAGG CAAAAGTAAA AACTGCAAAG CGTCTTAAAA    180

TAGAAGTTCA GAAGCCACAT TATATCACTT CTGTTGCATT CTATCAAAGC AAGTCACAAG    240

CCCCTGCCAA TCA                                                       253
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 183 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CACACACTCC CCCATTCTGA GCCCCAAGAG GCTCATCCCT AAGGATGTCC AGAGATCCAA 60

GTGCAGAAGG AGAATGTGGT GAGGCTATTT ATTCCCCCAG TGCCTTCCCT GCTGGGCTAT 120

GGATGAACAG TGGCTGACTT CATCTAGGAA AGAGCTATGG CTTCTGTCTC CTGGAGCTCA 180

CCA 183

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 387 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGTGAGCCCC AGGAGACAGA AGAGATATGA GGAAATTGTT AAGGAAGTCA GCACTTACAT 60

TAAGAAAATT GGCTACAACC CCGACACAGT AGCATTTGTG CCAATTTCTG GTTGGAATGG 120

TGACAACATG CTGGAGCCAA GTGCTAACAT GCCTTGGTTC AAGGGATGGA AAGTCACCCG 180

TAAGGATGGC AATGCCAGTG GAACCACGCT GCTTGAGGCT CTGGACTGCA TCCTACCACC 240

AACTCGTCCA ACTGACAAGC CCTTGCGCCT GCCTCTCCAA GGATGTTCTT ACAAAATTGG 300

TGGTATTGGT ACTGTTCCCT GTTTGGCCGA ATTGGAAAAC TGGTGTTCCT CCAAACCCCG 360

GTTATGGTGG GTTTCCTCCT CCTTGGA 387

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 366 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGGCGGAACA AGGGAGCGCT AAAAGGAAAT TAGGATGTCA GGTGCATAAA GGAACATAAT 60

TCCAAAACCT TTCCAAACCC CAAATTTATT CAAAGGAACT GAGGAGTGGA TTGAGGAGTG 120

GACCAACACT GGCGCCAAAC ACAGAAATTA TTGTAAAGCT TTCTGATGGA AGAGAGCTCT 180

GTCTGGGCCC CAAGGAAAAC TGGGTGCAGA GGGTTGTGGA GAAGTTTTTG AAGAGGGCTG 240

AGAATTCATA AAAAAATTCA TTCTCTGTGG TATCCAAGAA TCAGTGAAGA TGCCAGTGAA 300

ACTTCAAGCA AATCTACTTC AACACTTCAT GTATTGTGTG GGTCTGTTGT AGGGTTGCCA 360

GTTGTT 366

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 598 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTTGGGCCC CAAGGAAAAC TGGGTGCAGA GGGTTGTGGA GAAGTTTTTG AAGAGGTAAG 60

TTATATATTT TTGAATTTAA AATTTGTCAT TTATCCGTGA GACATATAAT CCAAAGTCAG 120

CCTATAAATT TCTTTCTGTT GCTAAAAATC GTCATTAGGT ATCTGCCTTT TTGGTTAAAA 180

AAAAAAGGAA TAGCATCAAT AGTGAGTGTG TTGTACTCAT GACCAGAAAG ACCATACATA 240

GTTTGCCCAG GAAATTCTGG GTTTAAGCTT GTGTCCTATA CTCTTAGTAA AGTTCTTTGT 300

CACTCCCAGT AGTGTCCTAT GTTAGATGAT AATGTCTTTG ATCTCCCTAT TTATAGTTGA 360

GAATATAGAG CATGTCTAAC ACATGAATGT CAAAGACTAT ATTGACTTTT CAAGAACCCT 420

ACTTTCCTTC TTATTAAACA TAGCTCATCT TTATATTGTG AATTTTATTT TAGGGCTGAG 480

AATTCATAAA AAAATTCATT CTCTGTGGTA TCCAAGAATC AGTGAAGATG CCAGTGAAAC 540

TTCAAGCAAA TCTACTTCAA CACTTCATGT ATTGTGTGGG TCTGTTGTAG GGTTGCCA 598

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGCCTCAGGC TGGGGCAGCA TT 22

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACAGTGGAAG AGTCTCATTC GAGAT 25

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGAGCTGCCT GACGGCCAGG TCATC 25

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GAAGCATTTG CGGTGGACGA TGGAG 25

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGCAAACTTT CACCTGGACT T 21

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTTGTGACTT GCTTTGATAG AATG 24

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GACAACATGC TGGAGCCAAG TGC 23

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACCACCAATT TTGTAAGAAC ATCCT 25

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGTCCAGAGA TCCAAGTGCA GAAGG 25

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GAGCTCCAGG AGACAGAAGC CATAG 25

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGGCCCCAAG GAAAACT 17

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TGGCAACCCT ACAACAGAC 19

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGGCCCCAAG GAAAACT 17

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TGGCAACCCT ACAACAGACC 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ACATTGAAGC ACTCCGCGAC 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AGAGTGGCAG CAACCAAGCT 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCCTCAGGCT GGGGCAGCAT T 21

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGTCACCTTC TGAGGGTGAA CTTGC 25

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

ACGACTCACT ATAAGCAGGA 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AACAGCTATG ACCATCGTGG 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ACGACTCACT ATGTGGAGAA 20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

AACAGCTATG ACCCTGAGGA 20

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GGAGCTGCCT GACGGCCAGG TCATC 25

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1599 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:115..744

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
GCGGCAGGCG CGGCAAATTA CGTTGCCGGA GCTGAACGGC GCGGCTGGTC TGAAGGCAAA       60

CAAGCGAGCG AGCGCGCGAT AGGGGCCGAG AGGACGCGCA GGTGGCGGCG TTGC ATG        117
                                                            Met
                                                              1

TCG CAC GGT CAC AGC CAC GGA ATG GGT GAC TGC CGC TGC GCC GCC GAA        165
Ser His Gly His Ser His Gly Met Gly Asp Cys Arg Cys Ala Ala Glu
                  5                  10                  15

CGG GAG GAG CCG CCC GAG CAG CAC GCC ATG GCT ACG CTG TAC CTG CGC        213
Arg Glu Glu Pro Pro Glu Gln His Ala Met Ala Thr Leu Tyr Leu Arg
             20                  25                  30

ATC GAC CTG GAG CGG CTG CAA TGC CTT AAC GAG AGC CGC GAG GGC AGC        261
Ile Asp Leu Glu Arg Leu Gln Cys Leu Asn Glu Ser Arg Glu Gly Ser
         35                  40                  45

GGC CGC GGC GTC TTC AAG CCG TGG GAG GAG CGG ACC GAC CGC TCC AAG        309
Gly Arg Gly Val Phe Lys Pro Trp Glu Glu Arg Thr Asp Arg Ser Lys
 50                  55                  60                  65

TTT GTT GAA AGT GAT GCA GAT GAA GAG CTT CTG TTT AAT ATT CCA TTT        357
Phe Val Glu Ser Asp Ala Asp Glu Glu Leu Leu Phe Asn Ile Pro Phe
                 70                  75                  80

ACG GGC AAT GTC AAG CTC AAA GGC ATC ATT ATA ATG GGA GAG GAT GAT        405
Thr Gly Asn Val Lys Leu Lys Gly Ile Ile Ile Met Gly Glu Asp Asp
             85                  90                  95

GAC TCA CAC CCC TCT GAG ATG AGA CTG TAC AAG AAT ATT CCA CAG ATG        453
Asp Ser His Pro Ser Glu Met Arg Leu Tyr Lys Asn Ile Pro Gln Met
        100                 105                 110

TCC TTT GAT GAT ACA GAA AGG GAG CCA GAT CAG ACC TTT AGT CTG AAC        501
Ser Phe Asp Asp Thr Glu Arg Glu Pro Asp Gln Thr Phe Ser Leu Asn
```

115 120 125

CGG GAT CTT ACA GGA GAA TTA GAG TAT GCT ACA AAA ATT TCT CGT TTT 549
Arg Asp Leu Thr Gly Glu Leu Glu Tyr Ala Thr Lys Ile Ser Arg Phe
130 135 140 145

TCA AAT GTC TAT CAT CTC TCA ATT CAT ATT TCA AAA AAC TTC GGA GCA 597
Ser Asn Val Tyr His Leu Ser Ile His Ile Ser Lys Asn Phe Gly Ala
150 155 160

GAT ACG ACA AAG GTC TTT TAT ATT GGC CTG AGA GGA GAG TGG ACT GAG 645
Asp Thr Thr Lys Val Phe Tyr Ile Gly Leu Arg Gly Glu Trp Thr Glu
165 170 175

CTT CGC CGA CAC GAG GTG ACC ATC TGC AAT TAC GAA GCA TCT GCC AAC 693
Leu Arg Arg His Glu Val Thr Ile Cys Asn Tyr Glu Ala Ser Ala Asn
180 185 190

CCA GCA GAC CAT AGG GTC CAT CAG GTT ACC CCA CAG ACA CAC TTT ATT 741
Pro Ala Asp His Arg Val His Gln Val Thr Pro Gln Thr His Phe Ile
195 200 205

TCC TAAGGGCTGG CCAAGGCTCC CATAGAGGCG CTGTGTCAGT GAAGATGTAC 794
Ser
210

GACTACCTGT TGGGAAGGAC AAAGGGATGA GGCTCCAGAG AGAGTTGGCT GCCACAGCTC 854

TGCCAAGCTT TGTCTTTGGG GCTTGCTGCA GAAACCTGGC CTACGGAAGA TACGACACCA 914

CTGGGAGGGT TGTGTAGGTG CCAGGGGACC ATCGTGGTTC TCTAGGGCGC TGTGGAAATT 974

GGGTCTTGGG CTGGGTGGCA TCTGGCAGTC ATGGGTAACA CTTGCTTTTC CAGTTAATGT 1034

GGCCATGTGA TTCCAAGTGT CATGTTGCTT TGTGGAAGAT TGTTGTGTGA CTTGTTTTTT 1094

TGATTTTGTA TTTGTTTTTT TAAAGGAAAC TATTTGTGGG CTATAGGAAA CTTTCTGATG 1154

CCTCCGGATT GTGTTAGTAG TAGCCATCAG GAGGGTCTCC AACTAAAACA CTTGTTCCTG 1214

CTTGCTCCTT TCCCCTCTCA TTGTTCAGCA TTCTTGTCAA GTTGCCCAGC TTGGAGTTGT 1274

CTGTCACGCA CATGTGTCCT GTGGTTATAG CTAGAAGGAC AGGAGTCTCC TGCTGATGCG 1334

TGATAGCTTA AGCTTGGGGA GAAGGTCTTT TCCACTGCCT AGCTAAGCAG TCTGGGGAGA 1394

GCATGGGGAT CATTTCTATG TGTGTGGGTA ATCTGGTCAG TAAGATTGAG ACTTAGTTAA 1454

GATTCCCCTT GGAAATTCCT TAATGTTTAT TAGCTTCTAA CTAGTGTTGT AAGTCCGATG 1514

CCAGAATTTG GAGATTTGAG TTCTTCTTTT CATGGCTTTT ATTCACTGTG ACTAATAAGC 1574

TTCCTAATAA ATCCTTGCCA GACTT 1599

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 210 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
Met Ser His Gly His Ser His Gly Met Gly Asp Cys Arg Cys Ala Ala
  1               5                  10                  15

Glu Arg Glu Glu Pro Pro Glu Gln His Ala Met Ala Thr Leu Tyr Leu
             20                  25                  30

Arg Ile Asp Leu Glu Arg Leu Gln Cys Leu Asn Glu Ser Arg Glu Gly
         35                  40                  45

Ser Gly Arg Gly Val Phe Lys Pro Trp Glu Glu Arg Thr Asp Arg Ser
     50                  55                  60

Lys Phe Val Glu Ser Asp Ala Asp Glu Glu Leu Leu Phe Asn Ile Pro
 65                  70                  75                  80

Phe Thr Gly Asn Val Lys Leu Lys Gly Ile Ile Ile Met Gly Glu Asp
                 85                  90                  95

Asp Asp Ser His Pro Ser Glu Met Arg Leu Tyr Lys Asn Ile Pro Gln
            100                 105                 110

Met Ser Phe Asp Asp Thr Glu Arg Glu Pro Asp Gln Thr Phe Ser Leu
        115                 120                 125

Asn Arg Asp Leu Thr Gly Glu Leu Glu Tyr Ala Thr Lys Ile Ser Arg
    130                 135                 140

Phe Ser Asn Val Tyr His Leu Ser Ile His Ile Ser Lys Asn Phe Gly
145                 150                 155                 160

Ala Asp Thr Thr Lys Val Phe Tyr Ile Gly Leu Arg Gly Glu Trp Thr
                165                 170                 175

Glu Leu Arg Arg His Glu Val Thr Ile Cys Asn Tyr Glu Ala Ser Ala
            180                 185                 190

Asn Pro Ala Asp His Arg Val His Gln Val Thr Pro Gln Thr His Phe
        195                 200                 205

Ile Ser
    210
```

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CTGGCCTACG GAAGATACGA CAC 23

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

ACAATCCGGA GGCATCAGAA ACT 23

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

AGCCCCGGCC TCCTCGTCCT C 21

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGCGGCGGCA GCGGTTCTC 19

CLAIMS:

1. A method for identifying markers for a disease state, comprising the following steps:

a) providing a first set of peripheral blood mRNAs from one or more subjects known to exhibit said disease state and a second set of peripheral blood mRNAs from one or more normal subjects;

b) amplifying both sets of mRNAs to provide nucleic acid amplification products;

c) comparing said sets of amplification products; and

d) identifying those mRNAs that are differentially expressed between normal subjects and subjects exhibiting said disease state;

wherein a difference in quantity of expression of an mRNA is indicative of a disease marker.

2. The method of claim 1, further defined as comprising the step of using said mRNAs as templates for DNA synthesis in a reverse transcriptase reaction.

3. The method of claim 2, wherein random hexamers, arbitrarily chosen oligonucleotides, promiscuous oligonucleotide primers, anchoring primers or a combination of these are used as primers in the reverse transcriptase reaction.

4. The method of claim 1, wherein arbitrarily chosen oligonucleotides, promiscuous oligonucleotide primers, anchoring primers or a combination of these are used as primers in the amplification step.

5. The method of claim 1, wherein the disease state is metastatic or organ confined cancer, asthma, lupus erythematosis, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, autoimmune thyroiditis, amyotrophic lateral sclerosis, interstitial cystitis or prostatitis.

6. The method of claim 5, wherein the disease state is metastatic prostate cancer.

7. The method of claim 5, wherein the disease state is metastatic breast cancer.

8. The method of claim 1, wherein said subjects are laboratory animals.

9. The method of claim 1, wherein said subjects are humans.

10. A method of detecting a metastatic cancer disease state in a subject, comprising the steps of:

a) detecting the quantity of expression of a metastatic cancer disease marker expressed in peripheral blood of said subject; and

b) comparing the quantity of expression of said marker in peripheral blood of said subject to the quantity of said marker expressed in peripheral blood of one or more normal subjects;

wherein a difference in quantity of expression of said marker in peripheral blood of said subject relative to quantity of expression of said marker in peripheral blood of said one or more normal individuals is indicative of a metastatic cancer disease state.

11. The method of claim 10, wherein said disease marker is an mRNA.

12. The method of claim 11, wherein said mRNA is amplified by an RNA polymerase reaction.

13. The method of claim 11, wherein said mRNA is amplified by reverse transcriptase polymerase chain reaction or ligase chain reaction.

14. The method of claim 10, wherein said detecting is by RNA fingerprinting, branched DNA or nuclease protection assay.

15. The method of claim 10, wherein said metastatic cancer disease state is metastatic prostate cancer.

16. The method of claim 10, wherein said metastatic cancer disease state is metastatic breast cancer.

17. The method of claim 11 in which said mRNA comprises one or more of the sequences or the complements of the sequences disclosed herein as Genebank Accession numbers D87451, T03013, X03558, M28130, Y00787, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:29.

18. The method of claim 10 in which said marker is a product of the interleukin 8 gene.

19. The method of claim 10, wherein said metastatic cancer disease marker is identified by the method of claim 1.

20. The method of claim 11, further defined as comprising the steps of

a) providing primers that selectively amplify at least a portion of said disease state marker;

b) amplifying said disease state marker with said primers to form nucleic acid amplification products;

c) detecting said nucleic acid amplification products; and

d) measuring the amount of said nucleic acid amplification products formed.

21. The method of claim 20 in which said primers are selected to produce an amplicon having a sequence of or complementary to a sequence of at least a 50 base contiguous segment of Genebank Accession numbers D87451, T03013, X03558, M28130, Y00787, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:29.

22. The method of claim 21, wherein said amplicon is from about 50 to about 500 bases in length.

23. The method of claim 21, wherein said amplicon is from about 100 to about 415 bases in length.

24. The method of claim 10, wherein said metastatic cancer disease marker is a polypeptide.

25. The method of claim 24, wherein said polypeptide is encoded by a nucleic acid sequence comprising the sequence disclosed herein as Genebank Accession numbers D87451, T03013, X03558, M28130, Y00787, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:29.

26. The method of claim 24, wherein said detection comprises antibody immunoreaction with said polypeptide.

27. The method of claim 26, wherein said detection comprises an ELISA, an immunoprecipitation, a radioimmunoassay, an immunohistochemical, Western blotting, dot blotting, or FACS analyses.

28. The method of claim 24, wherein said polypeptide is encoded by the IL-8 gene.

29. The method of claim 10 or claim 24, wherein said marker is a product of the IL-8 gene and wherein said comparison is between two alternatively spliced forms of an IL-8 gene product.

30. The method of claim 24, wherein the quantity of IL-8 polypeptide in peripheral blood is measured using an *in vitro* bioassay that detects at least one IL-8 mediated biological process.

31. The method of claim 29 wherein said markers comprise Genebank Accession # M28310, Y00787, SEQ ID NO:4 and SEQ ID NO:5.

32. A disease marker for prognosis or diagnosis of a disease condition, wherein said disease marker is identified by a process comprising:

a) providing a first set of peripheral blood mRNAs from one or more subjects known to exhibit said disease state and a second set of peripheral blood mRNAs from one or more normal subjects;

b) amplifying both sets of mRNAs to provide nucleic acid amplification products;

c) comparing said sets of amplification products; and

d) identifying those mRNAs that are differentially expressed between normal subjects and subjects exhibiting said disease state;

wherein a difference in quantity of expression of an mRNA is indicative of a disease marker.

33. The disease marker of claim 33, wherein the disease state is metastatic or organ confined cancer, asthma, lupus erythematosis, rheumatoid arthritis, multiple sclerosis, myasthenia gravis, autoimmune thyroiditis, amyotrophic lateral sclerosis, interstitial cystitis or prostatitis.

34. The method of claim 32, wherein the disease state is metastatic prostate cancer.

35. The method of claim 32, wherein the disease state is metastatic breast cancer.

36. The method of claim 32, wherein said subjects are laboratory animals.

37. The method of claim 32, wherein said subjects are humans.

38. A method of detecting prostate cancer in a biological sample, comprising:

(a) measuring the levels of IL-8 in combination with at least one prostate disease marker in said sample; and

(b) comparing said levels with corresponding levels obtained from reference populations of normal individuals, individuals with BPH and individuals with prostate cancer.

39. The method of claim 38 in which said prostate disease marker is selected from a group consisting of: total prostate specific antigen (PSA); prostate specific membrane antigen (PSMA=Folic Acid Hydrolase); prostate acid phosphatase (PAP); prostatic secretory proteins

(PSP_{94}); human kallekrein 2 (HK2); and the ratio of the concentrations of free and bound forms of PSA (f/t PSA).

40. The method of claim 38 in which the biological sample comprises peripheral human blood.

41. The method of claim 38 wherein the level of IL-8 in a biological sample is measured using at least one antibody that binds to at least one IL-8 gene product.

42. The method of claim 41 wherein the level of IL-8 gene product bound to antibody is measured by ELISA.

43. The method of claim 38 wherein the level of IL-8 in a biological sample is measured using at least one oligonucleotide probe that binds to at least one IL-8 messenger RNA (mRNA).

44. The method of claim 43 wherein the IL-8 mRNA is alternatively spliced to include intron 3.

45. The method of claim 43 wherein the level of oligonucleotide probe bound to IL-8 mRNA is measured by nuclease protection assay.

46. The method of claim 43 wherein the level of oligonucleotide probe bound to IL-8 mRNA is measured by RT-PCR™.

47. The method of claim 43 wherein the level of oligonucleotide probe bound to IL-8 mRNA is measured by ligase chain reaction.

48. The method of claim 43 wherein the level of oligonucleotide probe bound to IL-8 mRNA is measured by PCR™.

49. The method of claim 40 wherein the level of IL-8 in a biological sample is measured using an *in vitro* bioassay that detects at least one IL-8 mediated biological process.

50. The method of claim 44 wherein the level of IL-8 in a biological sample is measured using at least one molecule that binds to an IL-8 gene product, wherein said molecule is selected from a group consisting of: an IL-8 binding protein; and an IL-8 receptor protein.

51. The method of claim 48 wherein the level of prostate disease marker in a biological sample is measured using at least one antibody that binds to at least one prostate disease marker protein.

52. The method of claim 51 wherein the level of prostate disease marker protein bound to antibody is measured by ELISA.

53. The method of claim 39 wherein the level of prostate disease marker in a biological sample is measured using at least one oligonucleotide probe that binds to at least one prostate disease marker messenger RNA (mRNA).

54. The method of claim 43 wherein the level of oligonucleotide probe bound to prostate disease marker mRNA is measured by nuclease protection assay.

55. The method of claim 43 wherein the level of oligonucleotide probe bound to prostate disease marker mRNA is measured by RT-PCR™.

56. The method of claim 43 wherein the level of oligonucleotide probe bound to prostate disease marker mRNA is measured by ligase chain reaction.

57. The method of claim 43 wherein the level of oligonucleotide probe bound to prostate disease marker mRNA is measured by PCR™.

58. A method of differentially diagnosing prostate cancer and benign prostatic hyperplasia, comprising the step of measuring the levels of IL-8 in combination with at least one prostate disease marker in a biological sample.

59. The method of claim 58 in which said prostate disease marker is selected from a group consisting of: total prostate specific antigen (PSA), prostate specific membrane antigen (PSMA=Folic Acid Hydrolase), prostate acid phosphatase (PAP), prostatic secretory proteins (PSP_{94}), human kallekrein 2 (HK2), and the ratio of the concentrations of free and bound forms of PSA (f/t PSA).

60. The method of claim 59 in which said biological sample consists of peripheral human blood.

61. A kit for use in detecting a human disease, comprising:

(a) a pair of primers for amplifying a disease state marker consisting of a nucleic acid; and

(b) containers for each of said primers.

62. A kit according to claim 61 in which the pair of primers is selected to amplify a nucleic acid marker for metastatic human cancer.

63. A kit according to claim 62 in which the pair of primers is selected to amplify a nucleic acid having a sequence comprising at least a 50 base segment of Genebank Accession numbers D87451, T03013, X03558, M28130, Y00787, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:29.

64. A kit according to claim 62, comprising:

(a) a pair of primers selected to amplify a nucleic acid sequence comprising SEQ ID NO:4 or Genebank Accession # Y00787; and

(b) a pair of primers selected to amplify a nucleic acid sequence comprising SEQ ID NO:5 or Genebank Acccession # M28130.

65. A kit for use in diagnosing metastatic cancer in a biological sample, comprising:

(a) an antibody which binds with high specificity to a polypeptide having an amino acid sequence encoded by a nucleic acid sequence comprising Genebank Accession numbers D87451, T03013, X03558, M28130, Y00787, SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, or SEQ ID NO:29.

(b) a container for said antibody.

66. A kit according to claim 65, further defined as comprising:

(a) an antibody that binds with high specificity to a soluble IL-8 gene product;

(b) an antibody that binds with high specificity to a membrane bound IL-8 gene product; and

(c) a container for each antibody.

67. A kit according to claim 65, wherein said metastatic cancer is metastatic prostate cancer.

68. A kit according to claim 65, wherein said metastatic cancer is metastatic breast cancer.

69. A kit for detecting or differentially diagnosing human prostate cancer, comprising:

(a) at least one detection agent for measuring the levels of IL-8 in a biological sample;

(b) at least one detection agent for measuring the levels of at least one prostate disease marker in said biological sample; and

(c) containers for each of said detection agents.

70. The kit of claim 69 in which said prostate disease marker is selected from a group consisting of: total prostate specific antigen (PSA), prostate specific membrane antigen (PSMA=Folic Acid Hydrolase), prostate acid phosphatase (PAP), prostatic secretory proteins

(PSP_{94}), human kallekrein 2 (HK2), and the ratio of the concentrations of free and bound forms of PSA (f/t PSA).

71. The kit of claim 70 in which said detection agents are selected from a group consisting of: polyclonal antibodies; monoclonal antibodies; oligonucleotides; paired oligonucleotides designed to bind to opposite strands of a double-stranded DNA molecule; and at least one molecule that binds to an IL-8 gene product.

72. The method of claim 16 in which said breast cancer marker is selected from a group consisting of: SEQ ID NO:29 and Genebank Accession # D87451.

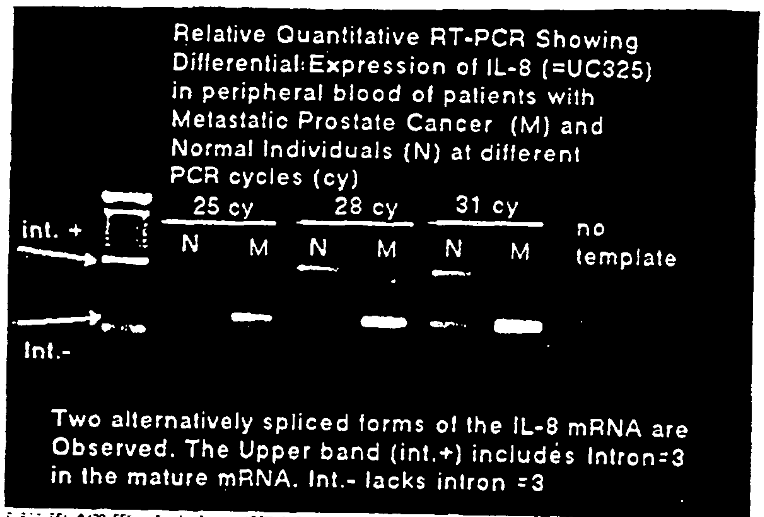

Relative Quantitative RT-PCR Showing
Differential Expression of IL-8 (=UC325)
in peripheral blood of patients with
Metastatic Prostate Cancer (M) and
Normal Individuals (N) at different
PCR cycles (cy)
25 cy
28 cy
31 cy
int. +
N M N M N M
no template
Int.-
Two alternatively spliced forms of the IL-8 mRNA are
Observed. The Upper band (int.+) includes Intron=3
in the mature mRNA. Int.- lacks intron =3

FIG. 1A

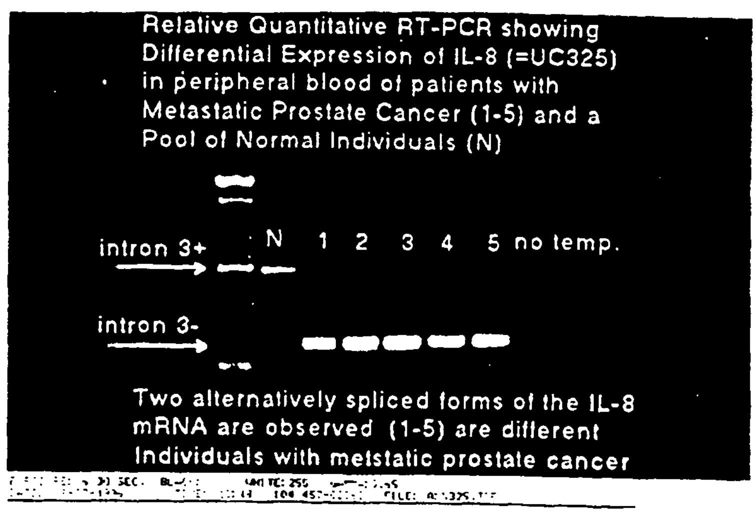

Relative Quantitative RT-PCR showing Differential Expression of IL-8 (=UC325) in peripheral blood of patients with Metastatic Prostate Cancer (1-5) and a Pool of Normal Individuals (N)
intron 3+
N 1 2 3 4 5 no temp.
intron 3-
Two alternatively spliced forms of the IL-8 mRNA are observed (1-5) are different Individuals with metstatic prostate cancer

FIG. 1B

Figure 2

Ability of Total PSA (ng/ml) to Distinguish BPH and Stages A, B, & C Prostate Cancer (n = 142)

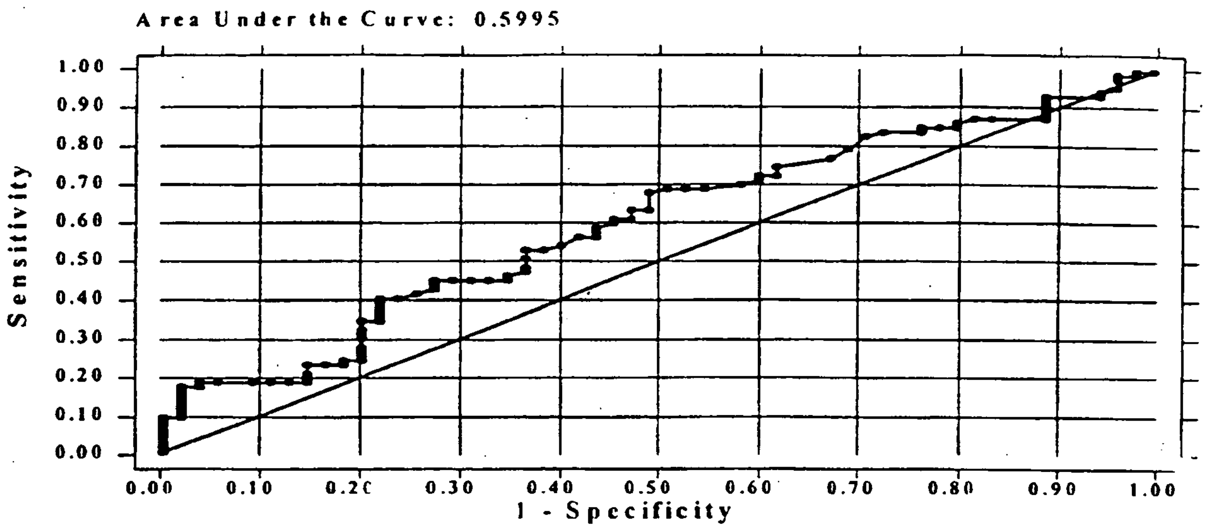


Figure 3

Ability of Corrected Free/Total PSA Ratio to Distinguish BPH and Stages A, B, & C Prostate Cancer (n = 142)

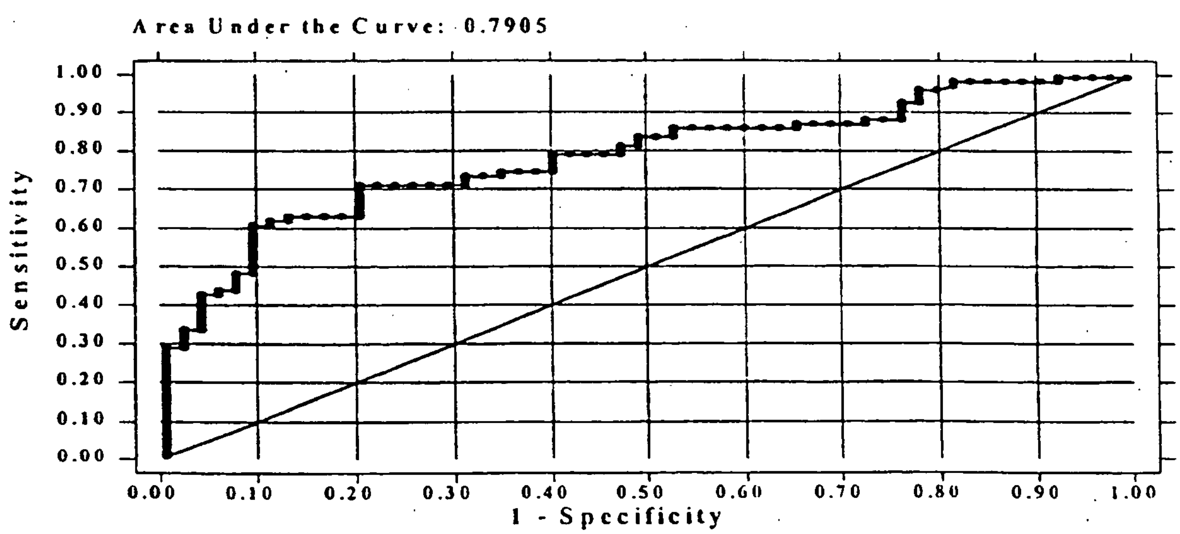


Figure 4

Ability of UC325 (pg/ml) to Distinguish BPH and Stages A, B, & C Prostate Cancer (n = 142)

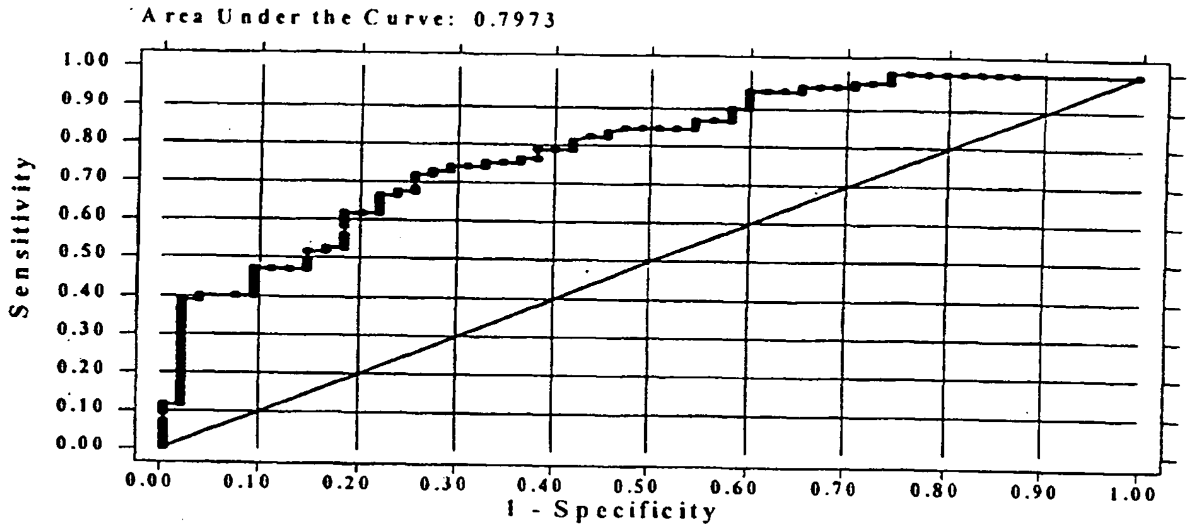


Figure 5

Ability of UC325 (pg/ml) & T-PSA (ng/ml) to Distinguish BPH and Stages A, B, & C Prostate Cancer (n = 142)

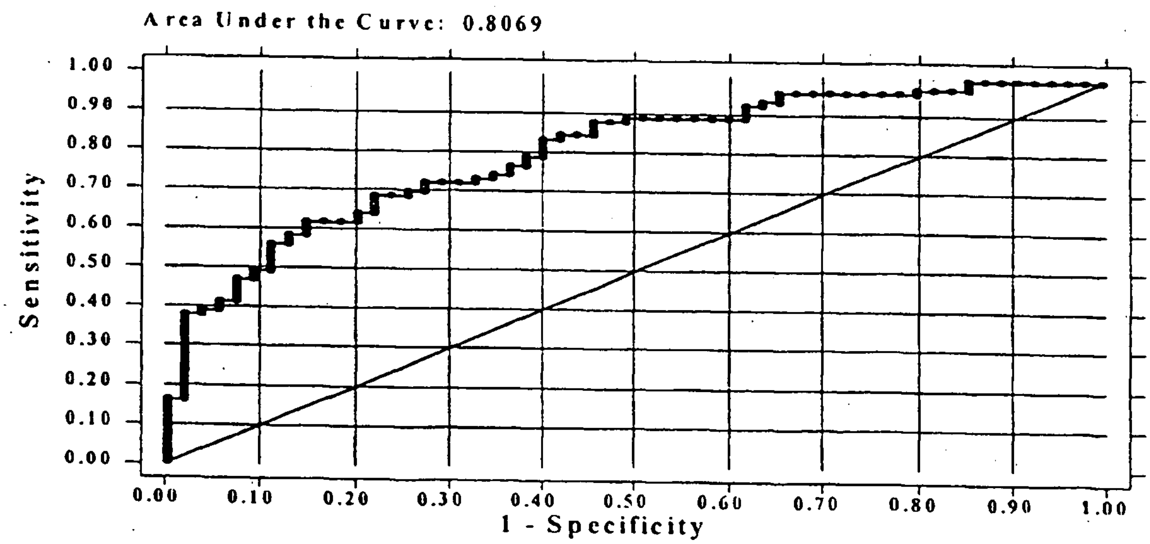
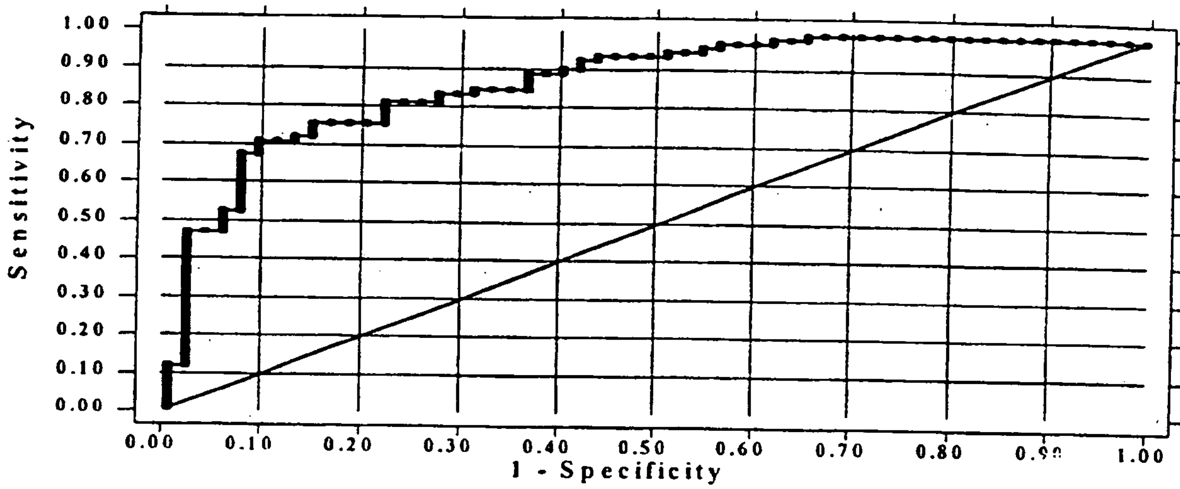


Figure 6

Ability of UC325 (pg/ml) & f/t PSA Ratio to Distinguish BPH and Stages A, B, & C Prostate Cancer (n = 142)

Area Under the Curve: 0.8784



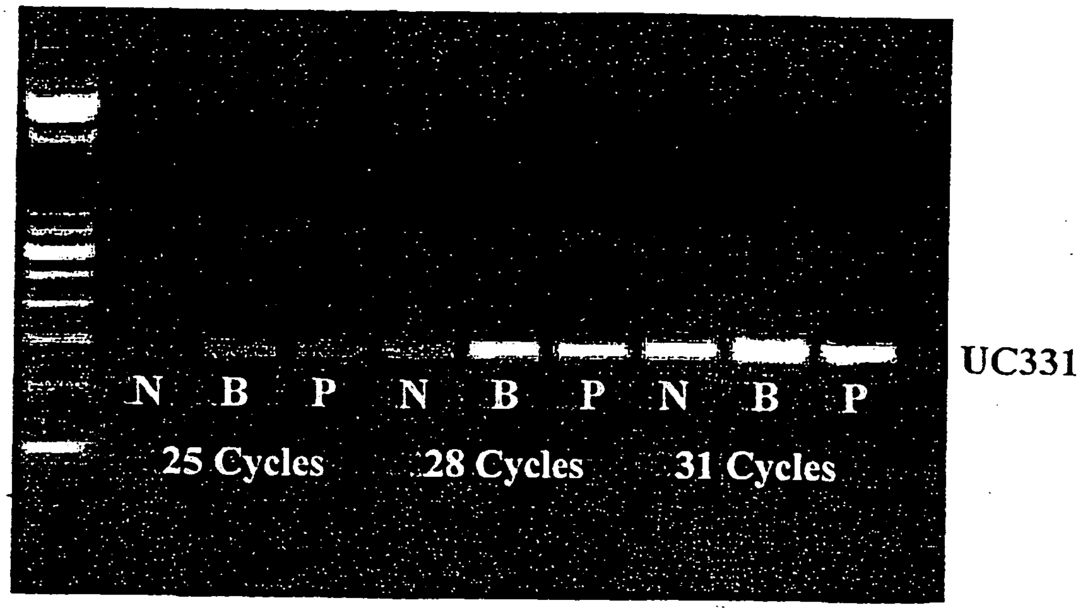


FIG. 7

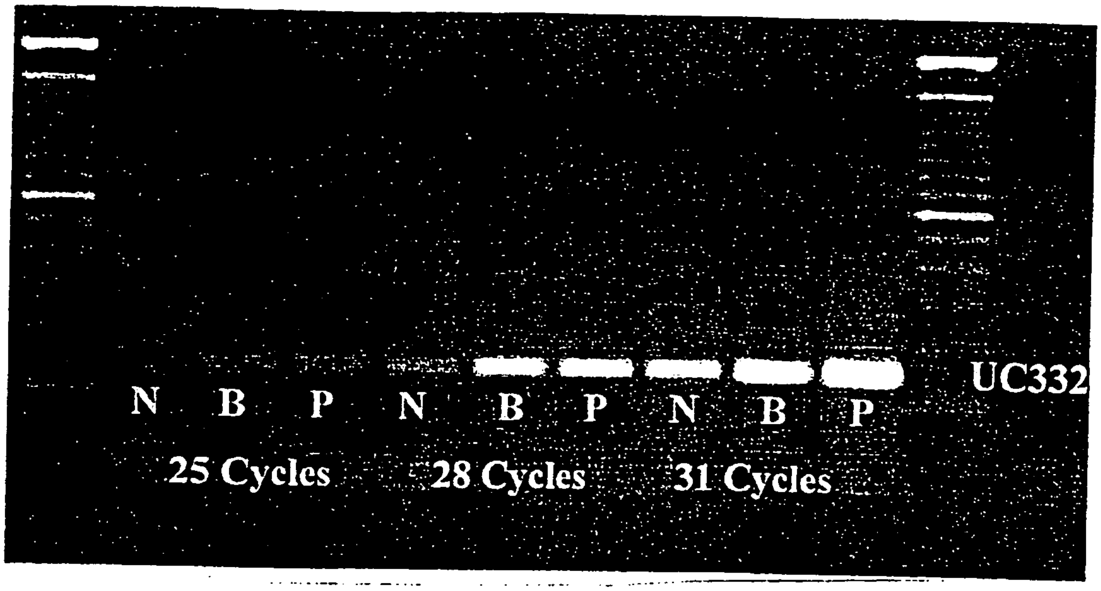


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22105

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/04; C07K 14/435; G01N 33/53
US CL :435/ 6; 7.1, 91.2, 91.21; 536/ 23.5, 24.31, 24.33; 530/350, 388.8
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/ 6; 7.1, 91.2, 91.21; 536/ 23.5, 24.31, 24.33; 530/350, 388.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,	US 5,539,096 A (BABAI et al) 23 July 1996, col. 2 and 5.	1-72
Y, P	US 5,677,125 A (HOLT et al) 14 October 1997, col. 7, 25, 26.	1-72
Y	US 5,236,844 A (BASSET et al) 17 August 1993, col. 5 and 7.	1-72
Y	US 5,459,037 A (SUTCLIFFE et al) 17 October 1995, col. 14-16.	1-72
Y	IVANOVA et al. Identification of differentially expressed genes by restriction endonuclease-based gene expression fingerprinting. Nucleic Acids Research. 1995, Vol. 23, No. 15, pages 2954-2958, especially pages 2954-2955.	14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 FEBRUARY 1998	20 MAR 1998

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHMID et al. Induction of mRNA for a serine protease and a B-thromboglobulin-like protein in mitogen-stimulated human leukocytes. The Journal of Immunology. 01 July 1987, Vol. 139, No. 1, pages 250-256, especially page 252.	17, 21, 24, 28-31, 63-65
Y,P	GREENE et al. Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an in situ messenger RNA hybridization technique. American Journal of Pathology. May 1997, Vol. 150, No. 5, pages 1571-1582, especially page 1580.	38-60, 69-71
Y, P	Database GenBank on STN, GenBank Accession No. D87451, NOMURA, N. 'Human mRNA for KIAA0262 gene, complete cds,' 10 July 1997.	72
Y	MATSUSHIMA et al. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. Journal of Experimental Medicine. June 1988, Vol. 167, pages 1883-1893, especially page 1886.	17, 21, 24, 28-31, 63-65

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/22105

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS; Dialog: Medline, CA, Derwent Patents, Biosis, Embase, GenBank
search terms: SEQ ID NO: 1-5, 29; mRNA, differential display or expression, cancer or tumor or metastasis